STUDIES ON THE GERMINATION OF BARLEY

by

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PREFACE

The material presented in this thesis consists largely of published papers reporting results of original work relating to various aspects of the metabolism of germinating barley. The writer performed or supervised all the experimental work and is the senior author of all but one of the papers included here. In that one paper (No VIII*) extensive reference was made to results obtained by my senior colleague, the late Professor I.A. Preece; the experimental work and the compilation of the paper were the responsibility of the present writer.

The collaborators in papers III and VII were undergraduate students who made minor, though important, contributions to the experimental work. Other collaborators (in Papers Nos VI, X, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX and XX) were post-graduate students who were working under my supervision. It is a pleasure to express my thanks to these students, not only for the work which they carried out with apparent enthusiasm, but also for the provocative discussions which they initiated and which not infrequently led to the genesis of new ideas.

Much of the work reported here is related to malting barley and thanks are due to the many maltsters who supplied samples of barley and of malt and who willingly discussed problems encountered in commercial malting.

* See pp. 3-5

Finally, I should like to record here the deep debt of gratitude I owe to the Late Professor I. A. Preece. His very genuine interest in this work, his encouragement at all times, his willingness to help in all possible ways and his own devotion to research set an example which one at least of his colleagues would wish to follow.

A.M.M.

PUBLICATIONS

I Studies on the Free Sugars of the Barley Grain. I. Historical Survey. J. Inst. Brew., 1952, 58, 270.

II Studies on the Free Sugars of the Barley Grain. II. Distribution of the Individual Sugar Fractions. J. Inst. Brew., 1952, 58, 363.

III Studies on the Free Sugars of the Barley Grain. III. Changes in Sugar Content during Malting (with D. C. Travis and D.G. Wreay). J. Inst. Brew., 1953, 59, 154.

IV Studies on the Free Sugars of the Barley Grain. IV. Low-molecular Fructosans. J. Inst. Brew., 1953, 59, 462.

V Raffinose Metabolism in Germinating Barley. New Phytol., 1957, 56, 210.

VI Comparative Studies of Embryo and Endosperm (with H. McCorquodale). J. Inst. Brew., 1958, 64, 112.

VII Cellulose Distribution in Barley (with J. P. Napier). J. Inst. Brew., 1959, 65, 188.

VIII Studies on the Free Sugars of the Barley Grain. V. Comparison of Sugars and Fructosans with those of other Cereals (with I. A. Preece). J. Inst. Brew.,

1954, 60, 46.

IX Barley Carbohydrate Metabolism in Relation to Malting. Wallerstein Lab. Commun., 1960, 23, 87.

X Lipid Metabolism in Germinating Barley. I. The Fats (with H. B. White). J. Inst. Brew., 1961, 67, 182.

XI Lipid Metabolism in Germinating Barley. II. Barley lipase (with H. B. White). J. Inst. Brew., 1962, 68, 487.

XII Water-soluble Carbohydrates of Seeds of the Gramineae (with H. McCorquodale). New Phytol., 1958, 57, 168.

XIII Trisaccharides of Lolium and Festuca (with H. McCorquodale). Nature, Lond., 1958, 182, 815

XIV Hemicellulases of Bromus Seeds (with R. Sandie). New Phytol., 1961, 60, 117.

XV Effects of Gibberellic acid on Barley Endosperm (with A. S. Millar). J. Inst. Brew., 1962, 68, 322.

XVI Ultra-structure of Caryopses of the Gramineae. I. Aleurone and Central Endosperm of Bromus and Barley. (with C. S. Johnston and J. H. Duffus). J. Inst. Brew., 1964, 70, 303.

XVII Gibberellic Acid in the Germination of Barley (with J. H. Duffus and A. S. Millar). Proc. Eur. Brew. Conv. Brussels, 1963, 85.

XVIII Development of Hydrolytic Enzymes in Germinating Grain (with J. H. Duffus and C. S. Johnston). J. Inst. Brew., 1964, 70, 521.

XIX The Embryo as an Activator of Gibberellic-acid-induced α-Amylase (with J. H. Duffus and D. J. L. Horsfall). J. Inst. Brew., 1966, 72, 36.

XX The Embryo of Barley in Relation to Endosperm Modification (with G. H. Palmer). J. Inst. Brew., 1966 (in press).

XXI On Barley. Trans. bot. Soc. Edinb., 1961, 39, 247.

XXII The Physiology of Malting. J. Inst. Brew., (in press).

INTRODUCTION *

For many years germinating barley (Hordeum vulgare L) has been a popular choice of plant material for biochemical and physiological investigation. One of the earliest studies of enzymic action in plants was performed with malting barley [5] and many subsequent students of seedling metabolism have apparently preferred to build on this foundation rather than start afresh with seeds of unknown potentialities. Moreover, barley is easy to grow on a laboratory scale and there is no difficulty in obtaining pure samples of known cultivars. Finally, the economic importance of barley for the brewing industry has resulted in continued practical interest being shown in the idiosyncrasies of germination of grain in bulk, and problems relating to the malting of barley suggest themselves almost annually.

The aim of malting is to produce germinated grain (malt) which, after milling and infusion in water at approximately 65°C. (mashing), will yield an extract containing the maximum amount of a suitably-balanced mixture of fermentable sugars and unfermentable higher saccharides. The formation of potential extract in malt

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* Arabic superscripts refer to references cited at the end of this Introduction.
Roman numbers, in brackets, refer to the publications listed on pp. 3-5 of the PREFACE.

is governed by the development in the grain of a range of hydrolytic enzymes, including hemicellulases, peptidases and amylolytic enzymes. The net action of the hemicellulases is to degrade the walls of the endosperm cells and thus modify the grain physically; a "well-modified" malt can be crushed so that the starch granules in the endosperm are liberated without breakage of the husks. The husks later form a filter-bed through which the enzymically-converted starch, and other solubilized grain components, are filtered to yield a malt extract, known as brewer's wort.

The formation of hydrolytic enzymes during malting appears to be a consequence of the interaction of gibberellin-like material with the aleurone cells of the endosperm (XV). Following the demonstration[29] that addition of gibberellic acid to barley during malting results in higher levels of amylolytic enzymes in the unkilned (green) malt, and allows adequately modified malt to be produced more rapidly, considerable interest has been shown by maltsters in the mode of action of gibberellic acid. Gibberellins occur naturally in barley[18,28] and the pattern of development of hydrolytic enzymes in the endosperm is substantially the same when barley is allowed to germinate without additives and when gibberellic acid is added to embryo-free endosperm (XVIII). It is therefore probable that the major changes taking place in the endosperm during malting depend largely on an enzyme-synthesizing site (the aleurone) and on an enzyme-inducing hormonal contribution

from the embyro (one or more gibberellins). Recognizing this, attempts have been made to produce malt with the aid of gibberellic acid from grain in which the embyro has been destroyed by irradiation,[23] by crushing[31] or by chemical means (XV). As malting loss, which is caused partly by respiration and partly by synthesis of unextractable seedling components, may amount to 10% of the dry weight of the original barley, the possible advantages of minimizing seedling metabolism are obvious.

The introduction of gibberellic acid to the malting industry has thus posed a number of problems which are of immediate relevance to maltsters who are interested in securing the benefits offered by gibberellins and at the same time concerned that the more economically produced malt should be a satisfactory brewing material.

A second physiological phenomenon of some complexity, which first seriously engaged the attention of maltsters during the last war, when there was a dearth of foreign grain in Britain, is the question of barley dormancy. The difficulties encountered at that time led to a comprehensive survey of grain storage, barley viability and dormancy being carried out[3] under the aegis of the Research Scheme of the Institute of Brewing. The cause of dormancy are not yet fully understood (XXII) but its importance is obvious to a maltster, who requires more or less simultaneous germination of over 95% of the corns in a sample amounting to

several hundredweights.

It is of interest to note that these two facets of plant metabolism - the mode of action of gibberellins, and the nature of dormancy - are inter-related, for gibberellic acid can overcome dormancy in barley (XXII), as it can in other seeds.[10]

Although problems encountered by maltsters may prove to be of general biological interest (and there is no doubt that gibberellin action and dormancy both have implications well beyond the malting industry) the converse is also true; work performed simply to extend knowledge of barley metabolism may have considerable repercussions on malting practice. A simple example of this comes from some investigations carried out by Kirsop & Pollock[19], who were interested in assessing the relative contributions of embyro and endosperm to the enzymic constitution of malt. Their results showed that, after three days' growth, the developing seedling had no further effect on the formation of α-amylase in the endosperm - and this observation, which can now be explained in terms of gibberellin action, led to the commercial use of root-killing steeps[13] which allow the production of easily manoeuvrable rootless malt of high potential extract. Not all work carried out to satisfy the curiosity of the investigator has such immediate practical application, but there can be little doubt that results of fundamental studies of barley germination and seedling growth

must ultimately form the basis of a proper understanding, and control, of the malting process.

The work reported in this thesis has been carried out against the background of the manufacture of malt, though the aspects of malting which have been studied were chosen in the hope that the results would contribute to fundamental knowledge, rather than to immediate technological progress.

SUGARS AND OLIGOSACCHARIDES OF BARLEY AND MALT.

Although the pioneer workers of the last few decades of the nineteenth century (notably Kjeldahl,[20] O'Sullivan[21] and Horace Brown[5]) laid the foundation of present knowledge of the distribution of free sugars in barley and in malt, a major advance in the understanding of carbohydrate metabolism in germinating barley came, in 1940, from the work of A.L. & W.O. James.[15,16] This work, which, along with the earlier studies, is surveyed in (I) was carried out before rapid methods of separating organic constituents of plants were readily available, and the introduction of paper chromatography and of micro-methods suitable for estimating individual sugars suggested the possibility of an extensive study of the vicissitudes of the simpler carbohydrates of barley during malting. Barley for malt is normally grown at a temperature of approx 14°C., and the accumulation of carbon dioxide and restriction of water on the malting floor after 3 days' growth might be expected to result in a different pattern of sugar distribution from that reported by James for a barley grown aerobically, with adequate moisture, at 21°C. An exploratory survey showed that paper chromatography, using butanol:acetic acid:water, and phenol:water as solvents, gave adequate separation of the sugars in the ungerminated grain. As would be expected from the earlier studies[15,16] the sugars

present in barley included fructose, glucose, sucrose, maltose and raffinose; additionally, a series of low-molecular fructose-containing oligosaccharides was detected. The simplest member of this series (apart from sucrose) was a trisaccharide, a glucodifructose. Ten samples of barley, representing four different cultivars, were analysed (II) and, though the absolute values for the individual sugars varied from sample to sample, sucrose constituted approximately 50% of the extracted sugars, raffinose 25% and the glucodifructose 13%. Over 75% of the sucrose and raffinose was restricted to the embyro and the aleurone layer of the grain. When the fluctuations in the free sugars of Ymer barley during malting were followed (III) it became clear that sucrose, raffinose and other fructose-containing oligosaccharides were utilized during steeping and in the early days of seedling growth, and that, although sucrose was re-synthesized as growth continued, raffinose could not be detected after three days' growth. Products of amylolysis appeared after 3 days, but sucrose remained the predominant free sugar. More recently, Edelman, Shibko & Keys[9] have shown that glucose can be transformed in the barley scutellum into sucrose by a system involving uridine diphosphate glucose; this doubtless accounts for the preponderance of sucrose in the germinating grain.

The methods used in this work involved treatment of the ground grain with 80% ethanol, and this solvent would not achieve

quantitative extraction of higher oligosaccharides. A survey was now made of the fructose-containing oligosaccharides by using aqueous extracts of enzyme-inactivated grain (IV). Column chromatography, on charcoal-kieselguhr, and the use of Whatman 3MM paper, allowed the separation of six separate bands of material, and, after hydrolysis with invertase and re-partition, the ratio of glucose to fructose was determined by the anthrone reagent. Evidence was obtained for the existence of a discrete series of fructose-containing oligosaccharides, based on sucrose, with the successive members each containing one additional fructose residue. The most complex material separated (which was probably a mixture) had a ratio of glucose to fructose of 1 to 9.6. No further investigation of this group of oligosaccharides has been undertaken, but it is possible that they resemble the fructosan separated from barley leaves[2] and later found to contain β·2,6-linked fructofuranoside residues.[14] The pentose sugars, arabinose and xylose, made a transient appearance in the free state towards the end of malting (III). Their liberation from the endosperm, which at this stage is essentially an autolysing organ, is not unexpected in view of the reported activity of various pentosanases in germinating barley.[25] The pentose sugars did not accumulate in the grain, and it must be presumed that they are metabolized by the seedling. The pentoses are readily detected on paper

chromatograms by virtue of the pink colour which they develop with aniline hydrogen oxalate, but the other major component of the endosperm wall, β-glucan[26] yields degradation products which defy simple observational detection. The soluble dextrin fraction recorded during malting would be expected to contain both α-linked components derived from starch and β-linked oligosaccharides representing the degradation products of the cell-wall β-glucan. No attempt was made to relate the glucose-containing oligosaccharides to their parent polysaccharides, largely because the hemicellulosic cell-wall material was already under investigation by Preece and his collaborators in this Department.

One sugar which did merit further investigation was raffinose. The trisaccharide (α-D-galactopyranose (1→6)α-D-glucopyranose (1→2) β-D-fructofuranose) consists of sucrose plus a terminal galactose residue; it does not seem to have been recorded from the vegetative organs of barley, though it is not uncommon in roots and rhizomes of seed-plants in general.[11] Raffinose in barley is largely restricted to the embyro (II) when it may account for approximately 10% of the dry weight (V). Recent work by MacLeod & Palmer (unpublished) has indicated that the distribution of raffinose within the embyro is not homogeneous: the scutellum contains much less than the axis, and the roots appear to harbour the bulk of the raffinose.

To follow the fate of raffinose during germination, grain was incubated, aerobically and anaerobically, at 21°C. for a period of 24 hr. and excised embyros were examined for their contents of raffinose and sucrose. No utilization of raffinose was noted during the first 14 hr., but thereafter the corns maintained in air metabolized the trisaccharide rapidly, and, at 24 hr., raffinose could not be detected in the embyros. In anaerobic conditions, however, there was little evidence of utilization; treatment with cyanide to inhibit terminal oxidases also interfered with the metabolism of raffinose. It may be significant that the time at which raffinose consumption begun (14 hr.) coincides with the time of rootlet emergence and that, when no root growth occured, as in cyanide-treated or aerobically-cultured grain, raffinose utilization was impaired. These results have confirmed and extended those earlier presented by James,[15] but the main object of the study was to determine the fate of raffinose, and not merely the conditions in which it is metabolized. No characteristic breakdown products of raffinose, such as melibiose or galactose, could be detected and, although galacton increased during the course of the experiment, there was no evidence for direct synthesis of this polymer from the galactose residue of raffinose. The means by which raffinose is mobilized therefore remain unknown, though it is of interest that a sucrose-containing

trisaccharide remains intact in conditions which allow the complete utilization of sucrose itself.

CARBOHYDRATES OF EMBYRO AND ENDOSPERM

Since the work outlined above showed quite convincingly that the sugars of the barley grain are not randomly distributed, it now seemed desirable to extend the analyses to include some of the less abundant polysaccharides. As rather large amounts of starting material were required, pearl barley was used to provide endosperm and, in default of a suitable source of material from barley, a commercial preparation of wheat germ was chosed for comparison. The polysaccharides derived from the pure endospermic material by extraction in warm water and in sodium hydroxide resembled, in yield and in analysis, those earlier described by Preece & Mackenzie;[26,27] neither galactan nor polygalacturonic acid appeared to be present. The materials extracted from the embyro, however, gave a clear indication of the presence of both galactose and galacturonic acid in their hydrolysates, and the proportions of arabinose were very much higher than in the products of the endosperm. (VI). These results suggest the presence of pectic material, with associated araban, in the embyro, and its absence from the endosperm. Using standard methods for extraction, it proved possible to separate a crude pectin-rich

preparation, contaminated with nucleic acid, from the embyro only; after fractionation, an almost pure araban was also obtained, though it was not isolated. Staining with ruthenium red indicated that barley embyros also contained pectic materials, and, in the absence of direct analysis, it seems not unreasonable to extrapolate the results from wheat to barley.

The apparent absence of pectin from the endosperm immediately raises the question of the constitution of any intercellular material located between the starch-filled cells. Exploratory work with Bromus, which has unusually robust cell walls in the endosperm showed that in B. sterilis individual units of the endosperm could be separated from one another in sections mounted in water by very slight pressure on the cover glass; with B. mollis, however, treatment with papain was required to secure separation of the cells. Barley samples varied: some showed slight separation into blocks of cells after 24 hr. in sterile conditions, in water, while others responded only after treatment with papain. It would appear, therefore, that a proteinaceous matrix may on occasion cement the endosperm cells together, a suggestion which, incidentally, is in accord with the unusual cytological development of the tissue (VI).

The last of the carbohydrates whose distribution was studied in detail was cellulose (VII). Crude cellulose prepared from whole grains was found to contain approximately 8% of xylose

residues and 20% of mannose residues, in addition to the expected glucose; yields were of the order of 4-5% of the grain. When successive rubbings from the grain were analysed, it was found that the cellulose was largely restricted to the outer fractions; the husk, for example, contained nearly 30% of cellulose whereas the final pearled grain contained only 0.4%. As this pearl barley was contaminated with some of the tissues from the furrow (which is obvious as a dark line in the pearl barley used in broth) it was of interest to assess the potential contribution from this source; calculation suggested that this "furrow" tissue might yield 0.37% of cellulose. It seems, therefore, that true barley endosperm completely lacks cellulose and that its walls are exclusively hemicellulosic. This observation is of some practical and theoretical interest, as modification of barley during malting demands the elimination of the wall components, and claims[12] have been made for the participation of a true cellulase, resembling a fungal enzyme, in this process. A survey of the relevant literature (reported in (VII)) suggests that there has been some rather loose thinking on this question; there may indeed be a true cellulase, capable of degrading native cellulose in barley - but there seems to be no cellulose for it to degrade in the endosperm.

The high mannan content of the crude cellulose was surprising, and it could be assigned largely to the contribution from the husk,

as analysis of excised embyros indicated that here the crude cellulose consisted largely of glucose residues, with only a trace of xylose present in the hydrolysate.

CARBOHYDRATES OF CEREALS OTHER THAN BARLEY.

Barley malt does not form the only source of fermentable sugars for the brewing process; indeed, all the common cereals (wheat, rye, oats, rice and maize) have at one time or another been used as supplementary sources of potential fermentables. These cereal preparations are known as "mash-tun adjuncts", and the choice of adjunct depends almost entirely on economic factors, the most popular adjunct at present being classified (low-protein) wheat flour which may constitute up to 30% of the grist.[30]

With a reasonable background of knowledge of the simple carbohydrates of barley now available, a general study was made of sugars and oligosaccharides of other cereal grains (VIII). This work had two objectives: first, it seemed desirable to complete the survey of the ready-formed sugars which were added to the mash tun and, secondly, and of greater importance, it was thought that information derived from other cereals (partly by experiment and partly by correlating published results) might shed light on some of the unsolved problems of the carbohydrate metabolism of barley itself.

In the course of the work some interesting points emerged. It was found that rye, wheat and barley resembled one another not only in their contents of fructose-containing oligosaccharides but

also in the relative amounts of water-soluble pentosans; additionally these are the only cereals which contain appreciable amounts of β-amylase in the ripe grain. All the cereals (including maize) contained raffinose, and only maize lacked fructosans. The similarity observed in the carbohydrate constitution of wheat, barley and rye might be attributed to the close phylogenetic relationship of the three cereals, but this similarity did not extend to the soluble β-glucan, which is abundantly present only in barley (and in the unrelated cereal, oats) and almost absent from wheat and rye.

An instructive observation, first made by Colin & Belval[7] for oats, is that the fructosan content diminishes, almost to disappearing point, as the grain ripens; one wonders whether the status of any grain constituent (structural or enzymic) can be discussed meaningfully from the results of analyses at one point in time (e.g. harvest ripeness) when the development, from fertilization to seedling establishment, is a more or less continuous process, with different components possibly increasing or declining at different rates in different cereals. These consideration are discussed in some detail in (VIII).

CARBOHYDRATE METABOLISM AND MALTING PRACTICE.

As was noted earlier, the original work presented here was initiated in the hope that it might yield results which would add to fundamental knowledge of the physiology of germinating barley. In reporting this work, the opportunity was taken where possible to relate results to malting practice but, additionally, critical review articles have been published in various periodicals devoted to scientific aspects of brewing. One such review (IX) is included here which summarizes, for the benefit of technical maltsters, the work carried out on cereal carbohydrates, and discusses their significance in the malting process; others are available elsewhere.*

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*See, e.g. Carbohydrate Metabolism in Malting. A.M. MacLeod: Brewers Digest, March, 1962, 40.
Les Constituants Hydrocarbones Mineur de l'Orge. I.A. Preece & A.M. MacLeod: Brasserie - Malterie de Belgique, May, 1963.

LIPIDS AND LIPASES OF GERMINATING BARLEY

As little sustained work appeared to have been carried out on fat metabolism in malting barley, though there is evidence that barley may utilize fat as a respiratory substrate, during storage and possibly in the early stages of seedling growth,[15,16] it now seemed desirable to examine, at least cursorily, lipid metabolism in malting. Of the 2% of fat present in whole barley, approximately one-third was concentrated in the embryo. Reversed-phase chromatography (X) provided a satisfactory means of separating the constituent fatty acids, and in addition to linoleic (52%), oleic (28%) and palmitic (11%), small amounts of stearic, myristic and linolenic acids were included in the saponified material prepared from the ether-soluble fraction. The amounts of the major fatty acids present were almost identical with those reported elsewhere for wheat germ.[33] The extent of mobilization of reserve fat during and after germination varied according to the condition provided for growth. When excised embryos were grown on moist filter paper, in starvation conditions, the lipid content fell in 48 hr. from 100 mg. per 500 embryos to 19 mg. and remained more or less constant at this level for the next 24 hr.; in laboratory or commercial malting of entire grain, on the other hand, the depletion of fat ranged from a mere 4% of the total to approximately 40%. Free fatty acids did not accumulate during

malting, so presumably, when they are produced, they are rapidly metabolized.

Lipase is undoubtedly present, even in ungerminated grain (XI) and apparently it is associated in its distribution with the reserve fat in the embryo and the aleurone. Extraction of the enzyme proved troublesome, and not more than 15% of the total lipase could be brought into solution. The extracted component - from barley and from malt - had a fairly sharp pH optimum at 6.8, and it proved to be very susceptible to inactivation by heat, in aqueous solutions, at temperatures above 30°C. The enzyme retained in the ground grain, however, had a temperature optimum of 48°C., and lipase activity was detected in kilned malt which had been subjected to temperatures very much higher than this. To estimate total lipase activity in germinating barley, a fine grind and a carefully controlled content of moisture in the grist were required: determinations were then made using triolein and tributyrin as substrates. Lipase activity in the entire grain fell during the first three days of seedling growth and then rose, rapidly in conditions of aerobic growth at 20°C. and more gradually in a commercial malting.

This work on fat metabolism did not really yield results of great interest, though it did add a little to general knowledge of metabolism in the early stages of growth of the grain. A study

of fat metabolism throughout the brewing process has recently been initiated by Witt & Burdick,[37] who have related their findings regarding the fatty acids of wort to the results reported in (X). These workers are also interested in the unsaponifiable fraction, which, in the studies alluded to here, was merely recorded as a constant amount throughout malting. Although the present writer found the topic of fat metabolism to be rather a dull one, the results obtained from this survey have at least formed a starting point for a more intensive investigation by others whose interest it has managed to arouse.

It is now possible to make a very general summary of the constituents of the embryo of barley which have been determined during the course of these studies, rembering that the figures quoted were obtained at different times, and from different samples of grain. (TableI).

What is of interest here is the high proportion of the dry weight of the embryo which appears to consist of reserve material which, in suitable circumstances, can be readily metabolized by the developing seedling. One tends to think of the embryo of a graminaceous seed as essentially a meristematic axis, together with a cotyledon (the scutellum) which functions as a digestive and absorptive organ. As will appear later (XX) the scutellum is minimally involved in the production of hydrolytic enzymes,

TABLE I

APPROXIMATE ANALYSIS OF BARLEY EMBRYOS

Constituent	Contribution to Embryo (mg. per 100 embryos)	Authority
Sucrose	13	V
Raffinose	9	V
Easily-hydrolysed polysaccharide	11	V
Cellulose	7	1X
Lipid	15	X
Protein (N x 6·25)	41	B.F. Folkes & E.W. Yemm*
TOTALS	96**	

*New Phytol., 1958, 57, 106. Value for a low-nitrogen barley.

** The dry weight of 100 embryos is of the order of 98 mg.

though it is certainly capable of absorbing products of starch hydrolysis,[9] and it now appears that it may resemble other cotyledons in containing substantial food reserves. Work at present in progress suggests that both lipid and sucrose are more highly concentrated in the scutellum than they are in the axis, though raffinose, as has already been mentioned, preponderates in the rootlets. It would seem, then, that the embryo is provided with an immediate source of nutritive material, sufficient to form an adequate substrate for respiration and growth until such time as the secondary reserves of the endosperm can be brought into solution and translocated to the seedling. The suggestion has been made that lipids may contribute more or less directly to the cuticle of the coleoptile,[16] and it is tempting to relate the raffinose to the mucilaginous material surrounding the root cap, but no firm evidence is available in favour of - or against - either of these speculations. For the present, both lipids and raffinose must be regarded broadly as constituents of the reserves of the embryo which are matabolized rapidly only in aerobic conditions.

CARBOHYDRATES OF THE SEEDS OF OTHER MEMBERS OF THE GRAMINEAE

Since the analysis of the common cereal grains (VIII) had provided some useful information, it was considered desirable to extend this work to include other grass species - rather on the principle of "What do they know of barley who only barley know?" The investigation was restricted to the water-soluble carbohydrates, which were considered broadly in two groups - sugars and oligosaccharides extracted by 80% ethanol and polysaccharides soluble in water at 40°C. Most of the twenty-two species examined (XII) contained sucrose as the predominant free sugar, but two of the Festuceae (Lolium perenne and Festuca pratensis) proved to have, as their most plentiful free sugar, a trisaccharide which contained galactose, glucose and fructose, but which was chromatographically distinct from raffinose. A more detailed study of this trisaccharide (XIII) suggested that it might be α-D-galactopyranosyl-3-α-D glucopyranosyl-2-β-D-fructofuranoside, and examination of other species of Festuca indicated that a higher homologue, a tetrasaccharide, might also on occasion be present.

As regards the other oligosaccharides present in grass caryopses, particular interest was attached to the fructose-containing compounds, which predominated in Elymus arenarius and in Bromus sterilis, and to raffinose, which was present in

approximately three-quarters of the seeds examined, and, in most of these, was accompanied by stachyose.

Although the polysaccharides separated from about half of the grasses studied proved to be rather amorphous products of ill-defined nature, with high positive specific rotations, five species (Bromus sterilis, Dactylis glomerata, Festuca pratensis, Arrhenatherum elatius and Avena fatua) yielded generous amounts of fibrous polysaccharide of specific rotation approximating to -10° and resembling the β-glucan found in barley and in cultivated oats;[27] Agropyron repens and Elymus arenarius resembled rye in their high contents of pentosans. Two species appeared to be very different from all others: Nardus stricta which provided a water-soluble mannan, and Molinia coerulea, which was unusually rich in galactan.

At first sight these results appeared to constitute little more than a jumble of meaningless and rather ill-assorted facts and figures. However, when attempts were made to group the genera on the similarities of the nature of the carbohydrates in their ripe seeds, some points of taxonomic interest emerged. For example, it was quite clear that Brachypodium differed greatly from Bromus (sens. lat.) with which it is associated by Clapham Tutin & Warburg,[6] though Hubbard (personal communication) considers that the two genera are not closely related. Again, the novel trisaccharide mentioned above was found in all species of Lolium and Festuca which were available for study, so emphasizing the close

relationship between the genera (XIII), but other analyses suggested that Festuca ovina (sens. lat.) represented a somewhat anomalous species. Further examples of the phylogenetic implications of these results are mentioned in (XII) and (XIII).

Although these taxonomic considerations were rather in the nature of an unexpected bonus which was not looked for when the investigation was started, the original purpose of the study (to offset the undesirable consequences of limiting one's interest to a single species) was fulfilled. The analytical results showed that different, reasonably-closely related species might differ quantitatively, but not qualitatively, in their contents of sugars or polysaccharides, and there seemed to be no reason why the species with the maximum content of any interesting constituent should not be used as experimental material and the results then extrapolated, cautiously, to barley. As will be seen later (p.28) this assumption has proved in one case to be fully justified.

It was tempting to consider the possibility of extending this simple analytical work to include a greater range of grass species, suggested by various taxonomists as being worthy of study, but it was decided, abeit reluctantly, to restrict further work of this type to an extended study of the genus Bromus, which offered a means of examining the β-linked polysaccharides which also occur in barley, where they are important in relation to modification of the endosperm.

Five species of Bromus were investigated (XIV) and the constituent polysaccharides were arbitrarily divided into warm-water soluble, autoclave soluble (freed from -linked components) and alkali-soluble material. The total extracted hemicellulose accounted for approximately 15% of the grain and three-quarters of this material (including all the water-soluble fraction) was present in the husk-free grain. A crude cellulose, on the other hand, appeared to be largely concentrated in the husk and absent from the endosperm. In Bromus the extractable hemicelluloses of the caryopsis proper amounted to at least eight times as much as the residual cellulose. It would thus appear that the endosperm walls resemble those of barley in their general structure though the quantities of β-linked products which could be prepared from Bromus were very much greater than those from barley: this is doubtless correlated with the very thick cell walls of the wild grass. A β-glucan ($\alpha_{[D]}^{t} = 10^{\circ}$) was prepared from the more readily soluble fractions and, on the evidence of the propertions of pentoses and hexoses obtained in hydrolysates, the β-glucan represented about one half of the total hemicellulose.

Bromus mollis was further studied over 8 days of germination and seedling growth and changes in the polysaccharide fractions showed conclusively that the β-glucan underwent progressive depolymerization and eventually become degraded to a stage at

which recovery by precipitation was no longer possible. In 8 days the recoverable β-glucan fell from 8% to 2.3% of the grain, and it is difficult to escape the conclusion that this wall material forms an important substrate for seedling growth. Changes in the pentosans were more difficult to interpret, though here also a pettern of degradation with some apparent transarabinosylation, was apparent.

Examination of crude enzyme preparations showed that an endo-β-glucanase, capable of diminishing the viscosity of β-glucan, was highly active in B. mollis, together with an exoglucanase which was estimated by the production of reducing groups additional to those which would be expected from the activity of the endo-enzyme.[24] These glucanases showed a 16-fold increase over those of barley when they were compared on the basis of (yield of crude enzyme x activity). A pentosanase (endoxylanase) from Bromus showed an eight-fold enhancement over that of barley. Not only was the pentosanase capable of producing, in less than 8 hr., a series of oligosaccharides ranging from an apparent hexasaccharide down to monosaccharides but it also was able to solubilize some 40% of an insoluble pentosan within 48 hr. On the other hand, even the most active enzyme preparations were without apparent effect on cellulose.

In all major respects, therefore, Bromus behaved on a fashion similar to that already established,[24-27] with much greater difficulty, for barley and it would seem legitimate to chose Bromus seeds in preference to barley if any more detailed biochemical studies of β-glucan or pentosan metabolism are required.

One facet of the enzymic potentialities of Bromus was selected for further examination. This was the very vigorous power of trans-β-glycosylation shown by crude enzyme preparations. In examining incubation mixtures of crude enzyme + cellobiose for potential cellobiase, it was regularly found that the digests suffered a decrease, rather than the expected increase, in reducing power, and chromatographic analysis showed that the products of cellobiose conversion included a series of higher oligosaccharides. Some of these were tentatively identified from their chromatographic characteristics and from their behaviour on electrophoresis in borate and busulphite buffers. β-Linked disaccharides (gentiobiose and laminaribiose) and a range of tri- and tetra-saccharides were present after a few hours incubation of 1% cellobiose with the enzyme preparation, and, with more prolonged incubation, oligosaccharides of greater complexity, (up to hexasaccharide level) were recorded. Transglycosylation also occurred from the 1.3 linked disaccharide,

laminaribiose, and the β·1.6 linked disaccharide, gentiobiose, but there was no apparent reaction when the β 1.2 linked disaccharide, sophorose, was supplied, or when glucose was used as a possible substrate.

From the results of an extensive series of incubations carried out with concentrations of cellobiose ranging from 0.1% to 5% and for times between 1 minute and 24 hr., it was concluded that the first product of reaction was a trisaccharide which was hydrolysed to glucase and a disaccharide, β·1.4, β·1.3 or β·1.6 linked according to the type of linkage formed in the initial trisaccharide.

Bromus thus contains an enzyme system, which can, in vitro, induce the production of many different β-linked oligosaccharides, though it did not prove possible to demonstrate, as had been hoped, the production of fibrous polymers akin to the insoluble hemicelluloses, and it is difficult to relate these laboratory findings to in vivo metabolism of the seedling.

This work, which is incorporated in a Ph.D. Thesis[17] has not been published (though it was designed as a sequel to (XIV)) because an apparently identical system of transglycosylation was described by Anderson & Manners[1] from barley. To detect transglycosylation in barley, however, solutions of 20% cellobiose

were incubated for 48 hr. to yield two newly-formed oligosaccharides, and up to 18 days' incubation was used to secure adequate amounts for characterization of the products of transglycosylation. The formation of the same range of compounds from 1% cellobiose by Bromus preparations in one hour attests the very much greater potency of the wild grass seed in this type of reaction.

HORMONES IN RELATION TO ENDOSPERM MODIFICATION

In the work so far summarized, the enzymes present in germinating barley and responsible for alterations in the amount of their potential substrates in the grain have been treated as if they operated in isolation. This is manifestly a gross over-simplification and the mechanism involved in synthesis and control of the hydrolytic enzymes, which was largely ignored until a few years ago, is worthy of intensive investigation. Among the factors which control the production of hydrolytic enzymes, the gibberellins are of outstanding interest.

Work on the relation of gibberellic acid to barley metabolism was initiated in response to the demonstration that addition of this compound could increase both the amount of α-amylase in finished malt and the rate of its production during malting.[29] By 1960, when the work now to be described was started, there was an enormous corpus of information available on gibberellins in general,[32] records of their effects on stem elongation and on induction of flowering were accumulating, and the efficiency of gibberellic acid as a dormancy-breaking compound was under investigation - but little fundamental work on interactions of gibberellins with malting barley had been reported.

As a first approach, the effect of gibberellic acid on the balance of soluble and insoluble hemicelluloses in malt was examined and, though it was clear that the treatment resulted in more extensive breakdown of the insoluble components of the endosperm wall, this approach did not shed light on the means by which the altered balance was achieved. During the course of this work, it was apparent that the most significant changes occured during the early days of malting, and attention was directed to the initial effects of gibberellin. At this time (1960) important contributions were made by Yomo[38] and by Paleg[22] who showed, independently, that gibberellic acid could affect isolated endosperms. Yomo recorded increases in α-amylase and Paleg reported the enhanced formation of reducing sugars in endosperm separated from its embryo and supplied with gibberellic acid.

Following this lead, an experimental system was devised for studying early responses to gibberellic acid: this involved the use of endosperm slices cut from husk-free sterile grain, and incubated in aseptic conditions. It was soon found (XV) that the aleurone is the only region of the endosperm which responds to gibberellic acid, that aerobic conditions are required for response, and that the optimum temperature for the reaction is 30°C. The first group of enzymes to be studied was the

hemicellulases (endo-β-glucanases and pentosanases), and it became clear that formation of both types of enzyme in the aleurone occured only in response to gibberellic acid; concurrently with the production of hemicellulases, the formation of α-amylase was deduced from the appearance of starch-derived oligosaccharides such as maltotriose. Products of protein degradation accumulated in the incubation vessels, but inhibition of proteolysis did not cause any diminution in endo-β-glacanase formation. It appeared, therefore, that gibberellin did not act via its influence on proteolysis, which might have accomplished the release of the carbohydrases from a proteinaceous matrix. Gibberellic acid had no effect on pre-formed hemicellulases in solution, though a minor and rather variable effect on homogenates of aleurone was detected, and it seemed that a particulate system was required for any response to occur, and an intact cell suspension for any considerable formation of enzyme. Not only was it clear that aleurone is the tissue which responds to gibberellic acid but it was also found that translocation of a "modification-including stimulus" from the embryo took place through the aleurone only.

That the events set in motion by gibberellic acid in the aleurone are the major ones in relation to modification was demonstrated by the production of a "malt" (which analytically resembled typical brewing malt) by treating the grain with chloroform

and then supplying it with gibberellic acid (XV). The chloroform, judiciously applied, killed the embryo and left the aleurone sufficiently "alive" to reduce tetrazolium chloride - and to show the usual response to gibberellin.

We now know much of what gibberellic acid can do in malting, but how does it function? It was suggested (XV), as a hypothesis for exploration, that gibberellic acid might be acting to disrupt lysosomes. This suggestion was consistent with the observation that production of hydrolytic enzymes appeared to be the main response to gibberellic acid, and with the well-known fact that increase in these hydrolytic enzymes occurs with remarkable speed on the third day in a typical malting. However, a search for lysosomes, using de Duve's methods,[8] gave no indication of structures behaving in a lysosome-like manner, and electron microscopy (XVI), which exposed the complexity of the aleurone, also failed to provide evidence for organelles of this nature. The lysosome hypothesis has therefore been abandoned.

If hydrolytic enzymes are not released en bloc from a lysosome-like organelle, they might none the less be present, in an inactive or a bound form, in the aleurone - or they might arise by direct synthesis from amino acids or from peptides. The results of work in which inhibitors of protein synthesis, and of oxidative phosphorylation, were used in conjunction with different

levels of added gibberellic acid suggested that active protein synthesis was a necessary concomitant of the production of endo--glucanase by barley aleurone (XVII). Certain anomalies were apparant when higher concentration of gibberellic acid (5.75×10^{-4}M) were used in conjunction with the inhibitors and these have not yet been fully explained. However, the work of Varner et al.[34,35,36] very strongly suggests that the production of one characteristic hydrolytic enzyme - α-amylase - is by direct synthesis from amino acids; gibberellic acid is believed by Varner[36] to act at the DNA - RNA level.

The demonstration that gibberellins occur naturally in grass seeds[18,28] has suggested that the material which maltsters were using, warily, as an additive, is probably a natural constituent of barley, and the obvious deduction from the observations made in (XV) is that an endogenous gibberellin is translocated from embryo to aleurone as a prelude to the formation of hydrolytic enzymes. Material extracted from excised barley embryos behaved in similar fashion to authentic gibberellic acid on thin layer chromatograms and give a positive result in a biological assay (XVII): more convincing characterization of barley gibberellins has been accomplished by Radley.[28]

A further series of observations which supported the hypothesis that normal malting, without additives, follows the

same pattern as malting in presence of gibberellic acid, involved examining the time course of production of different hydrolytic enzymes - α-amylase, endo-β-glucanase, protease and acid phosphatase. (XVIII). Slices of endosperm developed measurable activity of the different enzymes 12 hr. earlier than did endosperm of grain growing without additives - but the increase in enzyme activity was broadly parallel in the two sets of material. Bromus, which is splendid experimental material for this purpose, behaved similarly.

When isolated aleurone was examined for its response to gibberellic acid, it was found that the production of α-amylase and endo-β-glucanase amounted to some 40% of that obtained from an equivalent amount of whole endosperm slices. Comparison of these results with those of other workers[36, 39] is difficult, because methods used to separate aleurone vary widely. Our results suggest that isolated aleurone is efficient in producing hydrolytic enzymes in response to gibberellic acid, but in the absence of the underlying cells of starchy endosperm, secretion of enzymes is minimal.

It also appeared (XX) that some contribution from the embryo, other than gibberellic acid, was required to allow the aleurone to manifest its full potential for secreting α-amylase, and, accordingly, a more detailed study of the effects of the embryo

on the aleurone was initiated (XXI). Results of this work have emphasized the importance of the remnants of aleurone tissue which adhere to the periphery of the scutellum, and it may be that the embryo proper contributes even less than the 10% of the total α-amylase postulated by Briggs.[4] Excision of various parts of the embryo suggested that the nodal region may be of outstanding importance in relation to the supply of gibberellin-like material, and, quite by chance, an inter-relationship of gibberellin-controlled metabolism and auxin (indolyl acetic acid) was demonstrated. At 10^{-11}M, indolyl acetic acid enhanced the production of α-amylase in response to gibberellin, and it is just possible that this may be the additional material, derived from the embryo (XX), which enhances the usual response to gibberellic acid. This work on the metabolism of the embryo is in its very early stages, but it shows signs of linking up with some of the previous studies on the distribution of various reserve materials within the grain (II) and (X).

CONCLUSIONS

In the course of a discussion on the mode of action of gibberellic acid, Varner[36] (who has produced some very compelling evidence to suggest that the action of the hormone is associated with the formation of specific m-RNA molecules) dismissed the possibility that indolyl acetic acid might be involved in gibberellin-enhanced α-amylase production, partly on the grounds that it "becomes a very complicated problem when you introduce hypothetical third partners in this thing."

Seedling metabolism, however, is a complicated business and Varner's statement illustrates rather well the two complementary approaches which may be made to its understanding. On the one hand, there is the need to define and elucidate individual reaction pathways and, on the other, there is the desire to understand the integrated whole: very broadly, one could suggest that the biochemist favours the first attitude and the biologist the second - and both, in the long run, are necessary.

In the earlier stages of the investigations discussed here, some fairly simple biochemical techniques were used to determine the nature, distribution and fate of different components of the barley grain. More recently the emphasis of interest has changed to cover the behaviour of the grain as a whole - a broadening of outlook which was possibly induced by the need to

present, to an audience of general botanists, a comprehensive account of the metabolic activities of the barley grain (XXI). Again, the need to review, for the benefits of maltsters, current understanding of the physiology of barley germination (XXII) has suggested several problems concerning the growth of the whole corn which require exploring. One such problem is the need for an appraisal of the many metabolic events which occur simultaneously in the barley embryo during the first 24 hr. after the grain has been supplied with water (XX) and a second (XXII) is the nature of dormancy in barley. Both of these topics might benefit more from a critical synthesis of the many observations made by others than from a new experimental approach.

The work reported here has possibly contributed a little to knowledge of the metabolism of malting barley, and a few minor problems have been solved, but, to quote from (XXI), :-

"The barley plant may have been grown as an agricultural crop for over seven thousand years, the grain may have been malted since prehistoric times and the barley plant may have suffered as the victim of innumerable laboratory experiments and yet, I suggest, we have learnt just enough about the germination of the seed to make it at present one of the most fascinating of botanical studies."

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

BY

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

I. HISTORICAL SURVEY

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Investigations relating to the simple carbohydrates of the barley grain have developed along three lines, *viz.*, simple analyses of the grain, changes in sugar concentrations during maturation of the plant, and changes associated with respiration in the germinating corns. Much of the earlier work failed to take into consideration the fructosan fraction, so that results for sucrose have, in general, been unduly high, and the recent recognition of fructose-containing compounds of low molecular weight emphasizes the necessity for further investigation of the oligosaccharides of barley. Separation of the sugars by means of paper partition chromatography, or on charcoal columns, allied to a suitable micro-method of analysis, should prove of value in a re-examination of the relationships of the free sugars in the grain of barley.

Introduction

In a critical discussion on the relationship of the plant to its environment, Nicol[32] has commented on the rather strange situation at present existing where more complete analytical figures are available for the composition of bracken and of seaweeds than for that of any of the major crop plants. While this statement is perhaps rather sweeping with regard to barley when the vast numbers of isolated papers scattered throughout many journals are taken into consideration, it is certainly true that the relevant information is so widely distributed that it is outside the range of all but the most omnivorous of readers, and there are certainly still many lacunae in our knowledge of the composition of barley. Although the free sugars of the grain may amount to less than 2% of the dry weight, their importance in the developmental physiology of the seedling is undoubtedly great, and their disposition, concentrations and inter-relationships in barley are certainly worthy of continued and detailed study. While this has been appreciated for a very long time, and while sporadic attempts have been made to determine the precise part played in the grain by each sugar, analytical difficulties have been very considerable; the introduction of improved methods for the separation, identification and estimation of the simpler carbohydrates should now facilitate a reassessment of many problems of sugar metabolism.

It must be emphasized that any investigation devoted to sugars, to the exclusion of all other grain constituents, cannot alone hope to explain the mechanism of germination. The part played by the free sugars in initiating growth in the embryo cannot wholly be separated from the influence of more complex carbohydrates or from the effects of the enzyme complement of the grain, and the utilization of these sugars in the embryo may be modified by difficulties of permeability or by the action of germination inhibitors; nevertheless, only when all *individual* aspects of constitution and metabolism are understood will it be possible to assess the implications of each factor contributing to successful germination. A consideration of the part played by sugars in the grain and seedling may thus usefully form a starting point for a more comprehensive investigation of all the inter-related phenomena of germination.

The grain of barley occupies an intermediate position between two generations of the plant, forming, as it does, the culmination of one year's growth and the starting point for the succeeding season. Its biochemical composition is determined largely by the heredity and environment of the

parent plant, and, normally to a lesser extent, by conditions imposed during post-harvest storage. The behaviour of the grain during malting or during germination in the field is likewise influenced by external conditions which are, to a large extent, controllable during malting, and by the internal factors determined by the ancestry and past history of the sample. A comprehensive survey of the composition of the barley corn should ideally take into consideration not only the state of affairs apparent in the grain at the time of germination but also the circumstances, both environmental and genetical, which have contributed to its character. No single worker can hope to carry out an investigation on as broad a basis as that outlined above, so that it is desirable to attempt to co-ordinate the results of studies made from very diverse points of view. During the last half-century, investigations of the simpler carbohydrates of barley have developed along three main lines: (*a*) identification and estimation of sugars and fructosans in the grain; (*b*) analyses of the carbohydrate composition of the barley plant up to ear formation and grain ripening; and (*c*) estimation of changes in the sugar content of the developing seedling, with special reference to respiratory mechanisms. It will be convenient in the first instance to discuss the literature under these three general headings.

Sugars and Fructosans in the Grain

By the beginning of this century the presence of free sugars in the developing ears of cereals, in the ripe grain and during the early stages of germination was fully established, and some attempts had been made to give a quantitative estimate of the different soluble carbohydrates present in the barley corn. In 1875 Kühnemann[24] had demonstrated the presence of sucrose in germinating barley and Kjeldahl[21] later reported that, while ungerminated barley contained 1·5% of sucrose, green malt contained about five times this amount. According to Muntz,[30,31] who was amongst the first to recognize the importance of soluble carbohydrates in developing ears of cereals, sugar-like material represented approximately one-half of the substance of unripe grain, being replaced during ripening by starch. "Synanthrose," an ill-defined type of fructosan later shown by Colin & Belval[12] to have been contaminated by sucrose, was believed by Muntz to disappear from all cereals except rye during the ripening of the grain. Further studies on fructosans of cereal grains were initiated by Tanret,[37] who applied the term laevosine to a carbohydrate isolated from cereals. This substance was an amorphous powder of insipid taste, readily soluble in water and in dilute ethanol but almost insoluble in 95% ethanol. The specific rotation was −36°, and fructose was predominant amongst the hydrolysis products. For some years after these early records, however, the fructosans of cereal grains were unfortunately largely neglected, to the detriment of the accuracy of quantitative determinations of free sugars.

O'Sullivan[34] was the first to record the presence of raffinose in barley, and Frankfurt[16] ten years later detected a substantial amount of this trisaccharide in wheat germ. O'Sullivan[33] also confirmed the presence of sucrose in ungerminated barley and detected a reducing sugar mixture which had a lower specific rotation than that of glucose. Free fructose was therefore presumed to be present in the grain.

The classical study of germinating barley is that of Brown & Morris.[10] These workers showed the preponderance of sugar in the embryo of barley as compared with the endosperm; in grain which had been steeped for 48 hr. the embryo was found to contain 5·4% of sucrose and 1·8% of invert sugar, as compared with 0·3% of sucrose and 0·2% of invert in the endosperm. Maltose was not detected in ungerminated grain, but was present in the endosperm of barley which had germinated for 10 days. Brown & Morris further noted that excised embryos grown on a culture medium containing maltose accumulated considerable quantities of sucrose in their cells, while embryos supplied with glucose as a sugar source did not accumulate sucrose. The importance of sucrose was further illustrated by the results of feeding experiments with different sugars, sucrose proving by far the most successful nutrient for isolated barley embryos.

More than fifty years ago, then, it was realized that the ungerminated barley grain contained fructosans, raffinose, sucrose and reducing sugars, probably glucose and fructose. Sucrose was believed to be the most plentiful sugar, though the failure to make allowances for fructosans makes the figures

for sucrose not entirely unobjectionable, and both sucrose and hexoses were known to be more plentiful in the embryo than in the endosperm.

For the first quarter of this century little interest was shown in the free sugars of ungerminated grain. Perhaps the excellence and comprehensiveness of the investigations carried out by Brown & Morris may have led to the mistaken view that the function of the simpler carbohydrates in germination was fully understood—a point of view to which Brown & Morris would not have subscribed. Additionally, the fact that no major new techniques for extraction and estimation of sugars were evolved during this period must have discouraged more detailed studies. Of the sporadic papers that appeared, that of Kluyver[22] may be mentioned; sugars of the barley corn were estimated both by chemical means and by differential fermentation methods involving the use of different yeast strains. In the ungerminated grain, raffinose was found to account for 0·45% of the dry weight, sucrose for 0·76% and hexoses for 0·04%. Raffinose could no longer be detected after 24 hr. steeping and 24 hr. germination; maltose was not detected before three days germination. Ling,[26] however, has quoted very much higher figures for the free sugars of ungerminated barley, with invert sugar ranging from 0·56–1·43% of the dry grain and sucrose from 1·01–2·31%. The very different figures obtained by different workers may be due partly to varietal and seasonal fluctuations, and partly to varying methods of extraction and estimation; unfortunately, experimental methods are not always as fully described as could be desired. Once again, in all this work, no account was apparently taken of the possible effect of fructosans on the estimations.

Separation of the dry barley grain into its component parts presents considerable mechanical difficulties, as removal of the embryo from the endosperm is easily achieved only after the grain has been steeped for some hours; few records therefore exist of the distribution of sugars within the grain. Kretovitch,[23] however, established the fact that the concentration of sugars was not uniform throughout the endosperm of wheat, sucrose being five times more plentiful in the outer layers than in the inner. Moreover, weevil-infested wheat, which had been completely depleted of starch-containing cells, was found to contain no sucrose in the intact aleurone layer which had apparently suffered no damage through weevil activity. Whether these results are also applicable to barley remains to be seen. Finally, amongst the earlier investigations concerned with simple sugars in grain, reference must be made to the work of Bode.[8] In a genetical study of barley, the grains from some 200 plants were divided into two lots; one half was used for sucrose determinations and the other half was planted. This process was repeated for four years and it appeared that the sucrose content of the grain was largely independent of environment but was genetically controlled. Further, there was a tendency for the germinative capacity of the grain to be highest in samples with the highest sucrose content.

Other more recent investigations of the content of free sugars in the barley corn have dealt with changes in sugar concentration in relation to the respiration of the seedling, and will be considered later under that heading.

It has already been noted that, although the presence of a fructose polymer in cereals was reported by Muntz as early as 1878, little attention was paid to this fructosan for many years. Yet, as Archbold[2] has recently stated, "to omit fructosans from a quantitative study of sugars means disregarding up to half of the sugars present." However, in the early nineteen-twenties various French workers, notably Colin & Belval[11,12,13] and de Cugnac[14] re-examined the whole question of fructosans in cereals. Polysaccharides, predominantly fructosan in nature, were shown to be present in the ripening ears of wheat, barley, oats and rye, though from oats all fructosan had apparently vanished by the time the grains were ripe. No fructosan was detected at any stage in the ears of maize. Figures of the order of 0·5% dry weight are quoted for the fructosan content of barley grain; further analysis of barleys was not attempted, but in wheat the fructosan appeared to be present in the endosperm only, being absent from bran and germ. Space does not permit of a detailed consideration of the work carried out by the French school on fructosans, but a full and critical review of their findings is given by Archbold.[2]

The exact chemical constitution of the fructosan—or fructosans—of cereal grains

has not yet been fully established. Material isolated by Archbold & Barter[4] from barley leaves gave, on hydrolysis, a maximum of 95% fructose and, apparently, a small amount of glucose. Haworth, Hirst & Lyne[18] established the fact that this material resembled laevan from *Poa*, consisting as it did of a chain of fructofuranose units connected by 2 : 6 linkages; more recent analyses of fructosans from grasses (*cf.* Laidlaw & Reid[25]) have shown that many fructosans of this type contain a terminal glucose unit, probably linked with fructose as in sucrose. It is possible that the fructosan in the grain is of the same nature as that in the leaves, though no direct evidence seems to exist on this point.

Carbohydrate Composition of the Developing Plant

By means of a series of detailed studies of different parts of the barley plant, Archbold and her collaborators[1,3,4,5] have done much to elucidate the transformations undergone by the sugars and fructosans during vegetative growth and in the ripening of the ear. As had already been established by Yemm,[38] the sugars of the barley leaf comprise the usual trio, sucrose, glucose and fructose, accompanied in certain seasons by fructosans and a variable quantity of starch. Polysaccharides were absent from the leaves of plants which had received heavy dressings of nitrogenous fertilizers. Fructosan is normally present in the stem, where it represents a condensation product of excess sugars translocated from the leaves.

From a study of concentration data, Archbold[3] suggested that the sucrose synthesized in the leaf was translocated only to the relevant internode, undergoing hydrolysis at some stage of translocation and that the excess of hexose above the amount immediately required for respiration and growth was slowly converted to fructosan. It appears that each node, with its attendant sheath and leaf, acts as a unit, and that there is little evidence for a major degree of translocation of stored fructosan. The emerging ear is richly supplied with fructosan (60% of sugar may be in the form of fructosan, with 20% of sucrose and 20% of reducing sugars), and, while the soluble carbohydrates of the vegetative parts are of considerable importance in ear formation, the further development after emergence can be ascribed mainly to the activity of the flag leaf and its sheath and to the ear itself. A balance sheet drawn up by Porter, Pal & Martin[36] indicates that of the total carbohydrate of the ripe grain 25% is derived from assimilation prior to the emergence of the ear, 45% from assimilation by the flag leaf and its sheath, the top internode and the peduncle, and the remaining 30% from direct assimilation by the ear itself. The magnitude of the contribution from the ear and the relative unimportance of translocation are noteworthy. Apparently a similar state of affairs exists in wheat; Boonstra[9] found that, in wheat, only the top internode and the peduncle, together with the associated leaf, were of significance in ear-filling. The awns of barley spikelets make some contribution to carbohydrate synthesis for, as Harlan & Anthony[17] have shown, removal of awns at an early stage of development results in a diminished dry weight of the ear with an accompanying reduction in starch content.

It cannot as yet be said whether the carbon dioxide assimilated by the ear is preferentially synthesized to starch rather than to other products. The total sugars present in the ear at its emergence are, however, sufficient to account for night respiration during ripening, so that the soluble sugars of the ripe grain may represent the residue from the carbohydrate present at ear emergence. Although Belval[7] believed that fructosans in the grain of wheat were precursors of starch, Porter *et al.*[36] proved conclusively that, in barley, while the proportion of fructosan fell concomitantly with starch accumulation, the absolute amount of fructosan per ear rather tended to rise. A further example of the danger of placing too much reliance on changes in percentage composition when assessing the contribution of any single carbohydrate fraction to a growing and developing organ has recently been provided by Earley.[15] In maize, the concentration of sucrose falls rapidly during ear development, while starch accumulation is in progress. To the unwary this immediately suggests conversion of sucrose to starch, but when results are recalculated per ear, it is evident that the absolute amount of sucrose rises throughout the critical period. The desirability of presenting data which can be calculated as desired either on a percentage

basis or as absolute values for a given organ or plant is here made evident.

Although the overall picture of carbohydrate changes in the developing grain of barley is now moderately clear, many details are still obscure. Thus raffinose is known to be present in ripe barley, though no records seem to exist of its presence in the vegetative parts of the plant. It would clearly be of interest to determine at what point raffinose first makes its appearance in the developing ear. The fate of the fructosan reserves in the young seedling is of interest; indeed, innumerable problems concerned with the relationship of sucrose to fructosans, and of both to starch, suggest themselves. A full understanding of the possible range of sugar constituents in different samples of barley may well depend on a preliminary knowledge of possible variations in the developmental history of the parent plant, though varietal differences in sugar content are also worthy of consideration.

Free Sugars and Respiration

It is generally accepted that the free sugars of plants form a major respiratory substrate. Attempts have frequently been made to relate changes observed in the concentration of the various sugars to gas exchange and to respiratory rate, and thus to determine whether hexoses or sucrose or some other carbohydrate forms the preferred substrate for respiration. In an investigation of respiration in detached barley leaves, Yemm[38] was able to demonstrate that in the early stages of starvation, while the respiratory quotient was in the neighbourhood of unity, there was a rapid fall in sucrose (initially 60–70% of the total plastic carbohydrate in poor carbohydrate years), with concurrently a complete disappearance of fructose. Glucose rose initially and subsequently fell to a low value. Although no simple quantitative relationship could be established between the concentration of any individual carbohydrate and the rate of carbon dioxide production, it was suggested that utilization of sucrose took place preferentially from the fructofuranose moiety, glycolysis of glucopyranose being less rapid. In good carbohydrate years, fructosans were apparently unattacked for some 40 hr. after the leaf had been detached; thereafter the fructosan content rapidly fell concurrently with an interruption in the fall of sucrose, thus suggesting a possible fructosan → sucrose conversion.

In grain, a parallel to Yemm's studies on detached leaves may be found in work carried out with excised embryos. James,[19] in an analysis of seedlings from excised embryos grown in the dark on culture solution lacking carbohydrate, also found that sucrose diminished rapidly during starvation while raffinose fell rapidly at first and then more gradually. Hexoses, which were not further fractionated, were never abundant, but showed a slight tendency to rise after the sucrose had been virtually exhausted. Maltose was present in the free state both in the ungerminated embryo and during the first six days of growth, in concentrations of the same order as those of hexoses. When seedlings were excised from entire grains at different stages of growth, it was found that the raffinose of the embryo was completely consumed during the first 24 hr. and sucrose during the first 48; with the continued supply of carbohydrate from the endosperm, however, sucrose reappeared in the embryo though no further supply of raffinose was detected. Reducing sugars—maltose and hexoses—though quantitatively less than sucrose during the ten days of seedling development, rose steadily throughout. As supplies from the endosperm eventually ran short, sucrose concentration fell as it had done in the isolated embryos, though at a later date. Once again, the conclusion is reached that sucrose is of paramount importance as a substrate for respiration and for synthesis of cell-wall materials.

From results of a study of the emission of carbon dioxide from grains of barley germinating in the dark, James & James[20] concluded that, after an initial phase in which respiration depended on slow oxidation of fatty materials, sucrose and raffinose in the embryo formed the major respiratory substrates; on exhaustion of supplies in the embryo, endospermic reserves were hydrolysed, translocated and, principally in the form of sucrose, became available for respiration in the seedling. Coincident with the exhaustion of the embryonic sugar, there was a slight check in respiration, soon overcome, however, by deliveries of new respiratory substrate from the endosperm. These observations, taken in conjunction with the findings of Brown & Morris, suggest that a more detailed scrutiny of the relationships

between starch, maltose and sucrose might be of value. McCready & Hassid[27] have shown that barley seedlings can synthesize sucrose when supplied with glucose, fructose, mannose or galactose, the presence of hexose phosphate indicating that this type of synthesis involves a phosphorylase. Presence in barley grains of a phosphorylase participating in starch synthesis has been demonstrated by Porter[35]; it would be of interest to know to what extent phosphorylation is involved in the interconversion of sugars forming part of a pool of potential respiratory substrates.

Although the work carried out by James & James probably represents the most detailed study at present available of the utilization of sugars by the barley corn, a number of other investigations on similar lines are worthy of note. Barnell,[6] in investigating the carbohydrates translocated from the endosperm, was able to assess the relative amounts of hexose utilized in the embryo for respiration and for synthesis. The general course of respiration appears to resemble that later worked out in fuller detail by James & James, although as a result of the use of different methods of expression of results no more than a very general comparison is possible. A balance sheet of carbohydrate metabolism during seedling growth from 3–162 hr. at 22° C. in the dark (by which time some four-fifths of the carbohydrate reserves of the endosperm had been consumed) credits the embryo with a total gain of 1·44 g. hexose per 100 embryos, and a loss of 0·68 g. hexose through respiration. Loss from the endosperm was of the same order as seedling requirements, the intrinsic respiration of the endosperm being very low. Thus, of the carbohydrate delivered from the endosperm, approximately one-third was used in respiration and two-thirds in growth, contributing to formation of a seedling of 11 cm. shoot length. Though the nature of the sugar respired is at least provisionally established, it would be of interest to determine the identity of the fraction contributing to synthesis; here, obviously, a much fuller knowledge of the enzyme systems involved is urgently required.

Unfortunately once more, in most of this work on seedling respiration no account was taken of the fructosan fraction and, though details of the distribution of fructosans in the grain of barley have not yet been published, it is almost certain that they are not without significance in the metabolic changes of germination.

Recent Studies of Sugars in Barley

As was suggested earlier, analytical investigations of free sugars in grain became progressively fewer during the first 40 years of this century; this was partly because of the excellence of the earlier work, and partly through the lack of any notable improvements in technique. With the introduction of partition chromatography a much more refined method for detection and identification of trace quantities of sugars became available, and a number of preliminary studies have already been reported. MacLeod[28] detected in Spratt-Archer barley raffinose, maltose, sucrose, glucose and fructose and additionally a series of fructose-containing substances of low chromatographic mobility and an oligosaccharide believed to be a gluco-difructose. Montreuil & Scriban[29] also confirmed the presence of the known sugars and further recorded free galactose and traces of arabinose, xylose, ribose and uronic acids, and an oligosaccharide which was apparently identical with the gluco-difructose of MacLeod.

This work has as yet been only exploratory in nature and has added little to the great body of information available from the results of earlier investigations; the potentialities for future development are, however, great, and separation of sugars on paper or on cellulose or charcoal columns, allied with accurate micro methods for sugar determination, may resolve many problems. It is clear from the results of the preliminary studies mentioned that all the free oligosaccharides of ungerminated barley have not been identified, the fructosan fraction in particular being as yet very ill-defined.

In discussing the results obtained by earlier workers little attempt has been made to compare the very different concentrations of, for example, sucrose, variously reported for barley grains. Different analytical procedures would clearly extract different proportions of the fructosans of lesser molecular complexity which might be wrongly assigned to various recognized carbohydrate fractions, and it would be inadvisable to attempt to deduce too much from the recorded figures.

It will be of interest, however, to see to what extent the results obtained by means of the newer techniques agree with those obtained by traditional methods; if as great a volume of useful results is accumulated by the use of physico-chemical methods of separation as was by the efforts of the enthusiasts of the late nineteenth century, the increase in available knowledge of barley composition will be notable indeed.

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II

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

II. DISTRIBUTION OF THE INDIVIDUAL SUGAR FRACTIONS

BY

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

II. DISTRIBUTION OF THE INDIVIDUAL SUGAR FRACTIONS

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The free sugars of ten samples of ungerminated barley were separated by partition chromatography and estimated by the Somogyi micro-copper method. The absolute quantities of the different sugar fractions varied widely from sample to sample, but sucrose was always the most plentiful (mean values, 0.92% of grain, 49% of total free sugars) followed, in order of abundance, by raffinose and glucodifructose. Hexoses amounted to less than 10% of the total sugars.

In a sample of Spratt-Archer barley, approximately 75% of the sucrose and 80% of the raffinose was located in the germ and aleurone, whereas 65% of the hexose sugar was in the endosperm.

Introduction

In the first paper of this series,[14] an attempt was made to give a general account of the present state of knowledge of the origin, disposition, nature and eventual utilization of the free sugars of the ungerminated barley corn. Although raffinose, sucrose and hexoses have long been recognized as components of the grain, it has recently become increasingly clear that these are not the only sugars present; fructosans of low molecular weight and a trisaccharide now established as a glucodifructose[15] can readily be detected in chromatographic analyses of barley extracts, and the presence of these oligosaccharides suggests that the figures usually quoted for sucrose content of barley may require re-assessment. In view of the opportunities afforded by the use of chromatographic techniques, it seemed desirable to carry out a series of sugar determinations with different barley samples, and thus to provide a basis for a study of sugar metabolism in barley during malting. The present paper discusses a technique suitable for estimation of the small quantities of sugars likely to be encountered in plant material, and gives the results of a number of sugar determinations carried out on ten samples, representing five different varieties, together with a study of the disposition of the sugars within the grain of one sample of barley.

As a prelude to describing methods and quoting results, it is appropriate first to consider briefly some of the difficulties inherent in preparing plant material for analysis. Ideally, in the case of sugars, the final extract should contain all the free sugars from the grain and nothing but the free sugars; no degradation of polysaccharides with enhanced yields of low-molecular material should be possible at any stage of the preparation, and no contaminating material capable of interfering with the subsequent analytical methods should be present. These are counsels of perfection; to what extent can they be observed in actual practice, with reference to a specific problem?

Degradation of polysaccharides can be minimized by rapid enzyme inactivation, which is most easily accomplished by plunging the entire grain into boiling 95% ethanol and maintaining under reflux for 30 min. It is undesirable to inactivate enzymes after grinding the grain; it can readily be shown that there is a slight increase in the maltose content of barley which has been ground and stored prior to enzyme inactivation. Although hydrolysis by plant acids may represent a possible source of error, which can be obviated by the addition of a little barium carbonate to the extraction medium, this hazard was not encountered in the present study; the buffering capacity of the crude barley extract was apparently sufficient to prevent hydrolysis.

Either water or aqueous ethanol may be used as extractant for sugars; both have

advantages and disadvantages. The fructosans are easily soluble in water but not wholly so in the higher concentrations of ethanol so that, at first sight, aqueous extraction would appear to be the more efficacious method of preparing a solution of sugars from barley. However, it was found that reducing sugars were still present in the residual ground grain after four successive treatments with water at 37° C., whereas three successive extractions with boiling 80% ethanol under reflux sufficed to remove all detectable sugars. Again, the aqueous extracts, rich in fructosans, did not prove amenable to subsequent separation on paper chromatograms, and estimation of the very small quantities of monosaccharides present was rendered difficult by the preponderance of high-molecular fructose-containing material. The exhaustive treatment with 80% ethanol appeared to extract all mono-, di- and tri-saccharides together with an unknown proportion of the material of greater molecular complexity. The ethanol could subsequently be distilled off, leaving a convenient volume of an aqueous extract of barley.

Although this method of extraction appears to yield a solution containing all the desired material, without extraneous sugars derived from polysaccharide degradation, it cannot be said that the solution contains nothing but sugars. In the absence of a complete analysis of barley, it is impossible to state with any exactitude what contaminants may be included in such a preparation. Salts, amino acids and polypeptides in solution and lecithin and fats in suspension are all present, and their removal without affecting the sugars under investigation is a matter of some difficulty. The use of ion-exchange resins to produce a bright, salt-free solution would appear to offer a useful means of securing a relatively pure preparation of sugars; unfortunately, however, very large volumes of water are required to wash the sugar from the resin, and the subsequent concentration of the de-ionized solution results in a noticeable loss of sugars.

Methods which may be employed for clearing plant extracts prior to estimating sugars have been fully discussed by Archbold.[1] With aqueous extracts of barley stems, it was found that clarification by lead acetate or by charcoal caused a slight increase in copper-reducing value; these clearing agents were believed to remove material capable of interfering with the reducing power of the monosaccharides. As a slight reducing action was apparent after fermentation with *Saccharomyces cerevisiae*, Archbold also postulated the presence in the extracts of reducing substances other than sugars. In the present work with grain, charcoal clarification was also found to give a slight increase (*ca.* 5%) in copper-reducing power. However, as it was desired to develop a method applicable to barley at all stages of germination, it was felt that such clarification would be unwise; the possible formation of reducing oligosaccharides during malting had to be allowed for, and oligosaccharides tend to be preferentially adsorbed by charcoal. Lead acetate clarification may result in loss of fructose,[17] use of alumina, as recommended by James,[9] led to a loss of *ca.* 5% of the copper-reducing power of the extract, and none of the methods examined was effective in removing the fat-like material from suspension. Estimations of reducing sugars were therefore carried out with uncleared solutions. No allowance was made for possible interference by reducing substances other than sugars. The presence of substantial amounts of raffinose in the grain precludes the use of top-fermentation *Sacch. cerevisiae* for determining non-carbohydrate reducing material, and indeed, in view of recent studies by Harris, Barton-Wright & Curtis[8] any assumption that yeast will completely remove all reducing sugars from solution must now be looked on with suspicion.

Experimental and Discussion

Analytical Procedure Adopted

In the light of the considerations discussed above, the following method was finally adopted for the preparation of all barley extracts for sugar estimation:

50 g. of barley of known moisture content were dropped into 125 ml. of boiling 95% ethanol, maintained under reflux for 30 min., ground and returned to the same ethanol diluted to 80%. The ground barley was extracted three times with separate lots of 80% ethanol, the extracts were filtered through muslin and centrifuged free from starch and the ethanol was distilled off. The resulting aqueous suspension was stored overnight in the refrigerator, and the material which deposited was removed by

centrifugation. After concentration to about 50 ml., aliquots of this preparation were used, without further treatment, for estimation of total free reducing sugars. For determination of the individual sugar fractions, the remainder of the extract was concentrated to a syrup, which was thoroughly extracted with 90% ethanol for application to the starting lines of paper chromatograms. This procedure permitted extraction of all mono-, di- and tri-saccharides known to be present in barley, the residual insoluble material being of low chromatographic mobility. Considerable amounts of presumed fructosan were also extracted in the 90% ethanol, and were included in the subsequent analyses. The figures for "fructosans," as presented here, are of little significance; it is not known to what extent this type of material is soluble in the different concentrations of ethanol employed, and, in the present report, this "fructosan" fraction is simply regarded as a necessary concomitant of adequate extraction of the lower-molecular material. A further study of the fructosans will be presented in a later paper.

Partition was in butanol-acetic acid-water, and individual sugars were detected and washed from the appropriate area of the paper in the manner previously described.[13] All traces of sugar could be removed from the paper into less than 3 ml. water. A portion of paper of the same area as those supporting the sugars was cut from the region of the chromatogram which had been traversed by the solvent, but which was free of sugars. The washings from this strip were used as a blank in the sugar estimations. All determinations of individual sugars from the chromatograms and of total free reducing sugars in the extracts were made using the micro-copper reagent of Somogyi.[16] Reducing sugars from the chromatogram (glucose, fructose and maltose) were estimated directly; non-reducing sugars (sucrose, raffinose, glucodifructose and "fructosans") were hydrolysed with 0·5 N sulphuric acid before estimation. For neutralization, barium carbonate proved unsuitable as, with the very small quantities concerned, considerable losses of sugar were observed, and values as low as 70% of the true figure for a known amount of sucrose were recorded. When the acid was exactly neutralized by sodium hydroxide, however, over 95% recovery was possible, though in presence of excess alkali low results were again achieved. All estimations were recorded in the first instance as glucose equivalents. By the application of a conversion factor, based on the degree of polymerization of the sugar concerned and on the relative values for the Somogyi titrations of the monosaccharides comprising the molecule, absolute values for each sugar could be obtained.

The standardization curves for the Somogyi titrations were of the form $y = mx + c$ in the range $y = 0 - 4{\cdot}0$ mg. sugar; for glucose $y = 0{\cdot}145x + 0{\cdot}0026$. The constant c was slightly larger for fructose and galactose than for glucose and as c was ignored in computing the conversion factors there was a slight underestimation of sugars containing fructose or galactose. In the case of fructose, this underestimation was of the order of 0·7% when 0·10 mg. sugar was involved, with smaller percentage errors when larger amounts of sugar were taken. For the barley extracts, errors caused by ignoring c never amounted to more than 1% of the given value of the sugar concerned.

Standardization curves were prepared for the known sugars on several separate occasions, and substantially no variation was found with different batches of the Somogyi reagents.

Preliminary experiments had revealed that chromatographic separation, followed by this micro-copper method of estimation, gave a reasonable analysis of mixtures of reducing and non-reducing sugars in concentrations similar to those previously reported for barley. It was, however, soon realized that the results obtained for glucose and fructose in the present investigation were very much lower than those reported, for example, by Ling,[12] and a second series of analyses of known mixtures of sugars, in concentrations of the order of those obtained in the present investigation, was carried out. Table I gives the results of such an analysis, and illustrates the method used for calculating the absolute quantity of each individual sugar.

It can be seen that, although agreement is in general reasonably good, there is a significant under-estimation of fructose. Fructose is amongst the least rugged of the sugars and might suffer a certain amount of decomposition during partition and drying; moreover, fructose and glucose are the most mobile sugars of this mixture, and greater mechanical loss of these monosaccharides

TABLE I

DETERMINATION OF INDIVIDUAL SUGARS IN A MIXED SOLUTION

(For known concentrations taken see below)

(i) *Total reducing sugars determined directly:* Direct Somogyi determinations were carried out on suitably diluted aliquots of the solution. The reagents having previously been standardized against glucose, the glucose equivalents could therefore be calculated. In two parallel determinations, A and B, glucose equivalent values were obtained of, respectively, 242 and 250 mg. per 100 ml.

(ii) *Total reducing sugars from chromatogram:* The sum of the glucose equivalents of glucose, fructose and maltose (determination A, below) is 0·762 mg. True glucose in original solution is, therefore, 0·249 × 242/0.762, *i.e.*, 0·249 × 317·6. Similarly, the glucose equivalents of fructose and maltose in the original solution are given by 0·438 × 317·6 and 0·075 × 317·6 respectively. In determination B, the corresponding multiplier is 836·1.

(iii) *Non-reducing sugars from chromatogram:* With glucose equivalents determined after hydrolysis, the multipliers 317·6 and 836·1 are again applicable, true sugar concentrations being calculated by the aid of a conversion factor derived from standardization curves prepared for the individual monosaccharide units concerned and where applicable from the number and type of such units in each molecule.

Sugar	Glucose equivalents: From chromatogram (μg.) A	From chromatogram (μg.) B	Calc. to original solution (mg./100 ml.) A	Calc. to original solution (mg./100 ml.) B	Mean (mg./100 ml.)	Conversion factor	Sugar concentrations (mg./100 ml.): Found	Taken	Error
Glucose ..	249	90	79·1	75·2	77·1	1·00	77·1	79·2	−2·1
Fructose ..	438	177	139·1	148·0	143·5	1·06	152·1	167·4	−15·3
Maltose ..	75	32	23·8	26·8	25·3	1·77	44·8	43·0	+1·8
Sucrose ..	1888	626	599·6	523·4	561·5	0·98*	550·3	541·0	+9·3
Raffinose ..	583	235	185·2	196·5	190·8	1·03*	196·5	195·6	+0·9

* For error inherent in conversion factor, see text.

TABLE II

SUGAR CONCENTRATIONS IN DIFFERENT SAMPLES OF BARLEY

Barley variety	Moisture content (%)	mg. of sugar per 100 g. dry weight of barley: Glucose	Fructose	Maltose	Sucrose	Raffinose	Glucodifructose*	Fructosans†
1 Plumage-Archer 1950	11·2	29	114	52	909	553	246	331
2 Plumage-Archer 1950	11·0	43	68	113	1229	832	433	536
3 Spratt-Archer 1950 ..	10·8	86	36	20	356	262	214	163
4 Spratt-Archer 1950 ..	24·4	63	111	74	1008	627	326	306
5 Earl 1950	11·7	33	35	58	343	223	97	97
6 Ymer 1950	14·1	92	70	58	1110	333	243	485
7 Ymer 1950	9·4	62	53	66	1286	566	399	351
8 Carlsberg 1950 ..	8·9	38	33	135	572	144	110	178
9 Spratt-Archer 1951 ..	12·5	84	105	6	656	278	148	236
10 Ymer 1951	12·9	50	159	59	1690	772	339	460

* Conversion factor (*cf.* Table I) = 0·97. † Soluble in 90% ethanol.

would be expected to result from their relatively greater distances of travel. As the estimations made on known solutions showed values from 4–10% lower than the true value for fructose, and rather smaller positive or negative errors for the other sugars, it may be taken that the results for the barley extracts will have errors of similar magnitude. It is felt that the method is not yet sufficiently rigid to permit of the application of any correction factor for discrepancies of this nature.

Sugar Contents of Different Barley Samples

Table II gives a summary of the concentrations of the different sugar fractions in ten different samples of barley, analysed by the methods described above. Each set of figures represents the mean of at least two separate analyses, and all figures are calculated on a dry-weight basis.

Although the concentrations of the different sugars vary considerably from sample to sample, a number of general principles are apparent. Thus, in all the samples examined sucrose is the most plentiful of the sugars, followed in order of abundance by raffinose and glucodifructose; the hexose sugars are present in very low concentrations, of the order of 0·1% of the dry matter of the grain. There is a certain regularity of composition in the different sugar fractions, which can more easily be seen from Table III, where the separate sugar concentrations are expressed as percentages of the total sugar of the grain. With only ten analytical results available, it would be unwise to stress unduly this appearance of symmetry of constitution; however, in view of the principle of regularity of carbohydrate composition enunciated by Bishop & Marx,[3] it is certainly worthy of note. The regular disposition of sucrose, raffinose and glucodifructose (mean values respectively 49·1%, 24·6% and 13·9% of total sugar), apparent even in Sample 4 with 24·4% moisture, adds weight to the concept of a rather precise metabolic balance, acting to maintain a definite equilibrium amongst the different possible sugar fractions. In passing, it may be noted that the four Scandinavian barleys investigated had a somewhat different pattern of sugar distribution from that of the British varieties. The absolute amounts of sucrose varied over an equally wide range for both types of barley, but the Scandinavian barleys contained a mean value of 55% of the total sugar as sucrose, compared with 45% for the British barleys. Applying Student's *t* test to these values, it would appear that the difference between the means is significant at the 1% level. Unfortunately no information was available as to conditions of cultivation and storage, and the two types of barley may have differed not only in broad genetical characteristics but also in some other particulars not so easily detected. This statistical significance, therefore, may be more apparent than real, though it is in agreement with Bode's observation[4] that the sucrose content of barley appears to be genetically controlled.

While the existence of a symmetrical pattern of distribution amongst the sugars is, perhaps, the most interesting feature of these analyses, a number of other points are worthy of comment. Thus, the results for the hexose sugars are very much lower

TABLE III

INDIVIDUAL SUGARS AS PERCENTAGES OF TOTAL SUGAR CONTENT

(Fructosans not included amongst sugars)

Barley variety	Glucose	Fructose	Maltose	Sucrose	Raffinose	Glucodi-fructose
1 Plumage-Archer 1950 ..	1·5	6·0	2·7	47·8	30·0	12·3
2 Plumage-Archer 1950 ..	1·6	2·3	4·2	45·2	30·6	15·9
3 Spratt-Archer 1950	8·8	3·7	2·1	36·5	26·9	21·6
4 Spratt-Archer 1950	2·8	5·0	3·3	45·6	28·4	14·8
5 Earl 1950	4·2	4·4	7·6	43·4	28·2	12·3
6 Ymer 1950	4·8	3·7	3·0	58·2	17·5	12·7
7 Ymer 1950	2·5	2·2	2·7	52·9	23·3	16·4
8 Carlsberg 1950	3·7	3·2	13·1	55·4	13·9	10·7
9 Spratt-Archer 1951	6·6	8·2	0·5	51·4	21·8	11·6
10 Ymer 1951	1·6	5·1	2·9	54·5	25·0	10·9
Mean of all ten samples.. ..	3·8	4·4	4·2	49·1	24·6	13·9
Mean of British varieties (Nos. 1, 2, 3, 4, 5, 9) ..	4·3	4·9	3·4	45·0	27·6	14·7
Mean of Scandinavian varieties (Nos. 6, 7, 8, 10)	3·1	3·6	5·4	55·2	19·9	12·6

than those previously reported. Ling,[12] for example, found that hexoses accounted for 0·56–1·43% of the dry weight of the five barleys he investigated, while in the present study the comparable figures for ten samples of barley were 0·07–0·21%. Even if the maltose is incorporated with the hexoses as total free reducing sugars, the mean value is only approximately one-sixth of that estimated by Ling. It must be remembered, however, that all the samples here investigated were combine-harvested, and, with one exception (No. 4, Table II) were subjected to rather drastic drying, whereas the earlier work was presumably carried out on barley which had been allowed to mature in stook, and subsequently dried by less drastic methods. It is known that there is an increase of free reducing sugars during steeping, and that, during kilning, malt may suffer a loss of some 45% of the total reducing sugar present in the green malt. By analogy, it may be suggested that barley maturing in the stook may show a gradual increase in hexose content, comparable to the known increase in steeping, whereas rapidly-dried barley may suffer a loss of hexoses, comparable to that observed on kilning malt. This matter is now under investigation. Unfortunately Sample 4, which was received from the combine without drying, cannot be used for comparative purposes, as it had a distinctly mouldy odour at the time of analysis, and showed obvious growth of *Penicillium* and *Fusarium* some two or three weeks later.

Free maltose has rarely been detected in living plants, though a previous record of maltose in barley embryos does exist.[9] It is probable that the small quantity of maltose found in grain is an inevitable consequence of the presence of large amounts of starch together with active β-amylase. A certain degree of starch hydrolysis during ripening is rather to be expected; it is, perhaps, surprising that the values for maltose are as low as they are.

Little true comparison is possible between the concentrations of sucrose here recorded and those reported in earlier investigations, though it may be of interest to comment on and account for the obvious discrepancies. Thus Ling fround that sucrose varied from 1·01–2·31% with a mean of 1·51%, whereas in the present study sucrose varies from 0·34–1·69%, with a mean of 0·9%. It is almost certain that Ling, in common with all who carried out analyses of the sugars of barley before the introduction of chromatography, incorporated glucodifructose and possibly some of the fructosans of lower molecular weight in the estimations of sucrose, thus achieving unduly high results for the sucrose content. The range of the sum of the concentrations of sucrose and glucodifructose here observed is 0·57–2·03%. There is no means of deciding what proportion of the fructosans might have been returned as sucrose in the earlier estimations, but it would seem that the results here reported are of the same order as those previously recorded.

The mean value of the concentrations of raffinose (0·45% dry weight of grain) is identical with that quoted by Kluyver.[10]

The fraction here referred to as "fructosan" represents the fructose-containing material soluble in 90% ethanol and of lower chromatographic mobility than raffinose. The total amount of this fraction could be increased at will by decreasing the concentration of the ethanol used for final extraction of the syrup, without materially influencing the results for the simpler sugars. It must be understood that the figures given here are an expression of an arbitrary and incomplete extraction of the more complex oligosaccharides. It is probable that sucrose and glucodifructose are the two simplest members of this series of fructose-containing oligosaccharides (*cf.* the work of Bacon & Edelman with artichoke tubers[2]) but, in this preliminary study, the di- and tri-saccharides have been treated independently; their relationship to the polysaccharides will be considered at a later date when the high-molecular material has been more completely characterized. Naturally, no previous figures for concentrations of glucodifructose in barley are available as this tri-saccharide was positively identified in barley grain only recently.[15] It is interesting, however, to note that, from the results of chemical analyses, Archbold[1] was able to infer the presence in barley stems of sugar-like material intermediate in complexity between sucrose and fructosan.

Distribution of the Sugar Fractions within the Grain

Although the variations in the distribution of the different sugar fractions in different samples of barley are of some interest, a

TABLE IV

SUGAR CONTENTS OF THE DIFFERENT FRACTIONS OF THE GRAIN

Fraction of Grain	% of grain	mg. sugar per 100 g. dry weight of fraction						
		Glucose	Fructose	Maltose	Sucrose	Raffinose	Glucodifructose	Fructosans
1st Dust	11·5	—	—	—	—	—	—	—
2nd Dust	3·9	84	41	69	5277	3574	1325	274
3rd Dust	10·9	108	172	287	2999	1575	698	925
4th Dust	14·6	36	23	206	339	218	233	123
5th Dust	15·2	30	49	154	257	101	84	221
Pearl grain..	43·9	100	169	31	195	81	51	115

knowledge of the disposition of these same fractions within the grain is clearly essential for any detailed study of barley metabolism. The sugars present in the living regions of the grain—the embryo and the aleurone layer—may have a very different fate from those in the inert endosperm, and an understanding of the status of the soluble carbohydrates in the ungerminated grain is an essential prerequisite of any comprehensive picture of sugar relationships during malting.

A sample of Spratt-Archer barley (No. 9 in Table II) was used in this part of the investigation. The grain was subjected to laboratory milling for the preparation of pearl barley, and samples of the successive dusts and of the pearled grain were collected for analysis. After receipt, the samples were stored in air-tight jars during the period of the analysis, and no inactivation of enzymes was performed until work on the sample concerned was due to begin. Microscopic examination of the different dusts and of the rubbed grains at different stages gave an indication of the nature of each fraction, as follows:

1st dust: Mainly husk; with no significant amount of sugars present, no analyses were carried out with this fraction.

2nd dust: Aleurone layer and outer portions of embryo; 18 g. was used for analysis.

3rd dust: Mainly embryo, together with a little aleurone. This fraction was de-fatted with petroleum ether before extraction, 10 g. being used for analysis.

4th and 5th dusts: Sub-aleurone layers of the endosperm, together with aleurone from the edge of the furrow. 40 g. of 4th dust and 50 g. of 5th dust were used for analysis.

Pearled grain: Almost pure endosperm, with aleurone in the furrow. 100 g. were used for analysis.

The different weights of each fraction required for analysis are indications of the sugar contents, least material being taken for the portions of the grain containing most sugar.

The total amounts of the different sugars, expressed as mg. per 100 g. dry weight, are shown in Table IV. Since the relative amounts of the different fractions of the grain are known, it is possible to calculate the actual amounts of sugar present in each fraction, and the percentage distribution of each sugar in the different regions of the grain. It can be seen from Table V that, with the exception of maltose, the sum of the absolute amounts of each sugar in the different fractions is of the same order as the value for the intact grain. The very high figure for the sum of the individual fractions of maltose can probably be ascribed to starch hydrolysis during the milling and subsequent storage of the different dusts. The fact that the highest concentrations of maltose are found in the rubbings containing the inner region of the embryo and the aleurone and sub-aleurone layers, may be of some significance, for, according to Engel,[7] it is only these regions of the ungerminated grain which manifest any degree of β-amylase activity.

It is clear that the maximum concentration of the di- and tri-saccharides is in the fractions containing the aleurone and the

TABLE V

DISPOSITION OF THE SUGARS WITHIN THE GRAIN

Fraction	% of Grain	mg.* Glucose	% Total Glucose	mg.* Fructose	% Total Fructose	mg.* Maltose	% Total Maltose	mg.* Sucrose	% Total Sucrose	mg.* Raffinose	% Total Raffinose	mg.* Glucodifructose	% Total Glucodifructose
1st Dust ..	11·5	—	—	—	—	—	—	—	—	—	—	—	—
2nd Dust ..	3·9	3	4·6	2	1·8	3	3·0	206	28·9	139	35·4	52	26·4
3rd Dust ..	10·9	12	17·6	19	18·0	31	31·0	327	45·9	172	43·9	76	38·6
4th Dust ..	14·6	5	7·4	3	2·9	30	30·0	57	8·0	31	7·9	34	17·2
5th Dust ..	15·2	4	5·9	7	6·7	23	23·0	38	5·3	15	3·8	13	6·6
Pearl grain ..	43·9	44	64·7	74	70·5	13	13·0	85	11·9	35	8·9	22	11·1
Sum of fractions		68		105		100		713		392		197	
Intact grain ..		84		105		6		656		278		148	

* Calculated for a total of 100 g. grain (dry weight).

TABLE VI

MG. SUGAR PER 100 "EMBRYOS"

(Hexose equivalents)

Method of analysis	Hexoses	Maltose	Sucrose	Glucodifructose	Raffinose	Fructosans
Chromatographic method. This investigation. Spratt-Archer, 1951	1·32	1·2	12·6	2·94	6·75	*ca.* 4·11
Chemical method. A. L. James[9]. Plumage-Archer, 1934	1·04	0·58	15·26	—	5·86	—

germ, so that the concentration decreases from the periphery to the centre of the grain—a result comparable with that found for wheat by Kretovitch.[11] Thus (Table IV), the second dust (aleurone + outer layers of embryo) contains 5·3% of sucrose and 3·6% of raffinose, as compared with 0·19% and 0·08% in the endosperm. It may well be that raffinose is completely absent from the endosperm proper, and that the small amount detected has been derived from the remnants of the aleurone adhering to the furrow in the pearled grain. This would be in accord with the results of Colin & Belval,[6] who could detect no raffinose in barley flour, though they reported 4% of this sugar in the germ.

In contrast to sucrose and the other oligosaccharides, the bulk of the hexose sugars is found in the endosperm, and it would appear that this type of distribution persists in the early stages of malting; Brown & Morris[5] certainly found a similar preponderance of invert sugar in the endosperm after 48 hr. steeping.

It would be of interest to compare these results with a chemical analysis of the sugars of the different regions of the barley grain. Unfortunately no complete analysis appears to exist, and only a very general comparison with the results given by James[9] is possible, as his estimations are reported as mg. sugar per 100 embryos. However, taking into account the 1,000–corn weight and the moisture content of the fraction concerned, it is possible to re-calculate the values for the sugars in the 3rd dust on the basis of mg. per 100 corns content of that fraction—*i.e.*, very approximately as mg. per 100 embryos.

The figures thus obtained are shown in Table VI. This comparison is necessarily very crude, as the dust investigated contained substantial quantities of aleurone and of starch in addition to the germ. Moreover, the barleys concerned were different varieties

from different seasons and were grown under different environmental conditions. There can be no doubt, however, that with the exception of maltose, the relevant figures are of the same order of magnitude with the two very different methods of estimation employed; this is particularly marked when it is remembered that glucodifructose and some of the "fructosan" would have been included by James with sucrose. Certainly it seems clear that the results achieved by the use of chromatographic methods, rigidly applied, are as reliable as those obtained by the more laborious and time-consuming chemical techniques.

No useful purpose would be served at this stage by a detailed discussion of the results presented here. The method suggested appears to give reliable results, and, as complete analysis of the sugar content of a sample of barley can be carried out within a week, the technique should eventually yield results for many different barley varieties, grown and harvested under diverse conditions. Discussion will therefore be deferred until results can be presented for a wide range of samples, representing several seasons' crops; the principle of regularity of sugar distribution, the slight differences in the proportion of sucrose in different barley types and the asymmetrical distribution of the different sugar fractions within the grain indicated in the present work should then prove amenable to critical analysis.

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III

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

III. CHANGES IN SUGAR CONTENT DURING MALTING

BY

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COMMUNICATION

STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

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The concentrations of the principal free sugars and oligosaccharides of a sample of Ymer barley were estimated at approximately 24-hr. intervals from the commencement of steeping till the completion of kilning. Whilst sucrose, raffinose, glucodifructose and the lower fructosans generally diminished in steeping and on the first day on the floor, glucose and maltose contents during these periods showed little variation. Concurrently with the disappearance of raffinose from the germinating grain, there was a gradual accumulation of an oligosaccharide resembling maltotriose. The first appearance of free pentoses coincided with a marked increase in maltose content; glucose, maltose and the apparent maltotriose increased regularly during active growth on the floor. After the initial fall in sucrose, there was a rise in concentration till the final sucrose content was some five times that of the ungerminated grain. From a study of these malting changes it is suggested that oligosaccharides containing a glucopyranose-fructofuranose unit (sucrose, raffinose, glucodifructose and some fructosans) are intimately associated with respiration, while glucose, maltose and "maltotriose" primarily represent products of amylolysis. The relationship of the sugars produced by starch degradation to the predominant free sugar of the germinating grain—sucrose—has not yet been established.

Introduction

The traditional concept of plant growth as a metabolic process in which anabolism predominates over katabolism, with synthesis outstripping degradation, can be illustrated with peculiar appositeness in a germinating cereal seed. Not only are the two types of metabolic change proceeding simultaneously, but the chief locus of degradation is morphologically distinct from the actively growing and synthesizing region, the reserves of the endosperm forming the principal substrate for hydrolysis, with the embryo the centre of synthesis. During malting, breakdown of the carbohydrate of the endosperm shows as dissolution of the cell walls and pitting of the starch grains; changes associated with synthesis include formation of new cell wall material, both cellulosic and hemicellulosic, and deposition of starch in parts of the embryo. Concomitantly with these anatomically recognizable changes, there is a greatly increased rate of respiration in the developing seedling, maintained principally by the expenditure of relatively simple carbohydrates derived from both embryo and endosperm.

The sugars and oligosaccharides occupy a central position in the metabolic processes involved in germination. Following the partial dissolution of carbohydrates from the cell walls, hydrolysis of starch produces sugars which augment the limited supply in the embryo, and which are available both for respiration and for synthesis of new structural material in the plumule and the radicle. The content of free sugars in a living grain at any one moment is thus the result of a threefold dynamic equilibrium in which hydrolysis, respiration and synthesis each plays a part. It is unwise to allocate any one particular sugar to any one type of reaction simply from a consideration of concentration data relating to the different

sugar fractions present at different stages of germination; indeed, in view of the complexity of the reactions occurring in the living tissues, it is impossible to give a reliable account of carbohydrate metabolism by reporting detailed analyses of entire grains. Yet, on the other hand, to regard germination merely as the result of the sum of all the enzymatic changes known to occur during malting can, at present, produce only the most fragmentary and misleading of outlines. The most rewarding method of attack may be expected to involve both general analytical procedures and specialized studies of isolated enzyme systems.

It is desirable, however, that a thorough understanding of overall changes in sugar concentrations should precede specialized investigations of isolated and purified enzymes and their substrates; extraction methods not infrequently result in the juxtaposition of substances which are not normally associated with one another in the living plant tissue, and highly questionable conclusions may be drawn from test-tube experiments performed without reference to the organized metabolizing cell. For example, when extracts of germinated barley act on starch, considerable quantities of maltose and dextrins accumulate—yet, in the living grain, the principal disaccharide delivered to the embryo appears to be sucrose,[6] with maltose playing only a minor part. Again, phosphorylase appears to be inhibited by β-amylase in barley extracts[16]—yet starch is readily synthesized in the developing seedling. Such apparent anomalies are, in all probability, due to the uncontrolled nature of the reactions occurring in macerated tissue, in contrast to the organized activity of the living grain; they serve to emphasize the need to refer any reaction back to the living plant before asserting its importance in metabolism. Disintegrated plant tissues can carry out many interesting transformations, but it does not inevitably follow that these reactions are of great significance in the intact plant.

The present analysis of the free sugars of germinating barley was therefore designed to provide a basis for future, more detailed studies of individual aspects of carbohydrate metabolism during malting. The results can be expected to reveal only the residues and raw materials of the various metabolic processes; the nature of the changes taking place and the identity of the agents responsible for accomplishing these changes cannot be deduced with any degree of certainty from the results of a study of this nature.

Experimental

Germination at 21° C. (70° F.)

A sample of Ymer barley (germinative capacity 98·0%, moisture content 14·08%, 1,000 corn weight 42·04 g.) was selected for the preliminary laboratory analysis. 50 g. of barley were used for each period of germination, and both steeping and subsequent germination of the grain were at 21° C. in the dark, analyses being made to cover a range of 7 days, with 24 hr. intervals between successive tests. After 24 hr. steeping, without change of steep water, the grain was placed in a loose muslin bag and kept moist by regular sprinkling, with occasional turning. In this, and in all subsequent work, germination was considered to commence when the grain first came into contact with water; thus, a 3-day germination had actually undergone 24 hr. steeping followed by 48 hr. in a moist condition at 21° C. After the appropriate interval, enzymes were inactivated by means of boiling ethanol, and the grain was macerated in the ethanol used for enzyme-inactivation. Extraction and separation of sugars was carried out in general by the method previously described for raw grain[12]; with barley which had been germinated for 5 or more days, however, five successive extractions with 80% ethanol were necessary to ensure complete separation of the sugars from the grist. Growth at 21° C. was both uneven and rapid, an average length of 1·5 in. for the acrospire and of 1·0 in. for the rootlets being recorded after 6 days of germination.

The sugars present in this sample of Ymer barley proved to be similar to those previously observed[12] on chromatograms prepared from ungerminated barley. Neither galactose nor pentoses could be detected in the raw grain. Visual examination of the chromatograms suggested that no major change in the identity of the sugars present occurred during the first 3 days of germination; after 4 days growth, however, xylose and arabinose were recorded, though apparently only as minor constituents of the total free sugars. The presence of free galactose was also suspected simultaneously

with the unequivocal appearance of the pentoses; unfortunately, however, the faint spot which appeared on the galactose region of the chromatogram did not yield sufficient sugar for elution and re-partition in phenol-water. Both the presumed galactose and the pentoses appeared to be absent from chromatograms prepared after 5 days of germination.

Apart from the transient appearance of galactose and the pentoses, there was no obvious accumulation of any sugar not present in the ungerminated barley, nor was any sugar fraction completely depleted during the 6 days of germination. In the case of sucrose, maltose, glucose and fructose this was not unexpected, but the continued presence of an oligosaccharide with the same R_F value as raffinose was surprising, in view of the fact that James[6] had shown that all traces of raffinose disappear from Plumage-Archer barley embryos after some 3 days of germination. There is little[12] or no[1] raffinose present in the endosperm of barley, and the identity of the spot appearing opposite known raffinose therefore required careful investigation.

In extracts of ungerminated grain the spot with the same R_F value as raffinose could readily be shown to be, in fact, authentic raffinose; thus, it was non-reducing, it gave a strong ketose reaction with α-naphthol in phosphoric acid and on hydrolysis it yielded glucose, fructose and galactose. Examination of the oligosaccharide appearing opposite known raffinose on chromatograms prepared from barley which had been germinated for 6 days revealed that it gave a definite reaction with ammoniacal silver nitrate, thus indicating that it contained a free reducing group, and it failed to give a ketose reaction. This oligosaccharide, therefore, could not be raffinose. Although no unequivocal identification was achieved of the constituent monosaccharides produced by hydrolysis of this ketose-free trisaccharide, it appeared possible that maltotriose might be concerned. This trisaccharide has recently been detected in wort,[5] and its presence in grain during active amylolysis would not be unexpected. An authentic sample of maltotriose was partitioned against raffinose and against the trisaccharide from germinated barley in various solvent mixtures; while raffinose appeared to be slightly more mobile than maltotriose in butanol-ethanol-water, the barley trisaccharide gave the same R_F value as maltotriose in all solvent mixtures used. For the present, therefore, and until a further chemical analysis is available, it has been tentatively assumed that a trisaccharide resembling or identical with maltotriose is formed during the germination of barley.

The presence of the ketose-free reducing trisaccharide was not established until germination had proceeded for 6 days and raffinose had wholly disappeared from the seedlings; as it was probable that accumulation of maltotriose would overlap the gradual utilization of raffinose it was necessary to develop a method for estimation of maltotriose in presence of raffinose. To this end, the eluate from the raffinose region of the chromatogram was divided into two equal portions; one was used for a direct Somogyi estimation of free reducing sugar and the other portion was hydrolysed for estimation of the total glucose equivalent of the individual monosaccharides. Sufficient authentic maltotriose was not available for construction of a standardization curve; "maltotriose" from barley was therefore calculated as $2{\cdot}8t$, where t equals the glucose equivalent of the unhydrolysed trisaccharide. If the glucose equivalent of the hydrolysed moiety exceeded $3t$ the excess was allocated to raffinose, which was then computed by reference to a standardization curve for that trisaccharide. An approximate allocation of these trisaccharides as non-reducing (raffinose) and reducing ("maltotriose") could thus be achieved.

Results of the estimations of the individual sugar components of barley germinating at 21° C. in the dark are shown in Fig. 1. Calculation of these values was carried out in a manner similar to that used for the sugars of ungerminated grain, but each individual sugar was estimated directly from the titration values, by means of a standardization equation prepared for the sugar concerned, instead of by use of a conversion factor applied to the relevant glucose equivalent. By this means, the slight error due to ignoring c in the expression $y = mx + c$ was abolished[12].

As the presence of "maltotriose" was not established till an advanced stage in this series of determinations, no attempt has been made to estimate separately the trisaccharides raffinose and "maltotriose." Since maltotriose is a reducing sugar, the

values for the other reducing sugars are inevitably high in certain determinations, as the reducing power due to the trisaccharide has been proportionately divided amongst the known reducing sugars. This overestimation of glucose, fructose and maltose is significant only after 6 days of germination and does not affect the general trend of the sugar concentrations.

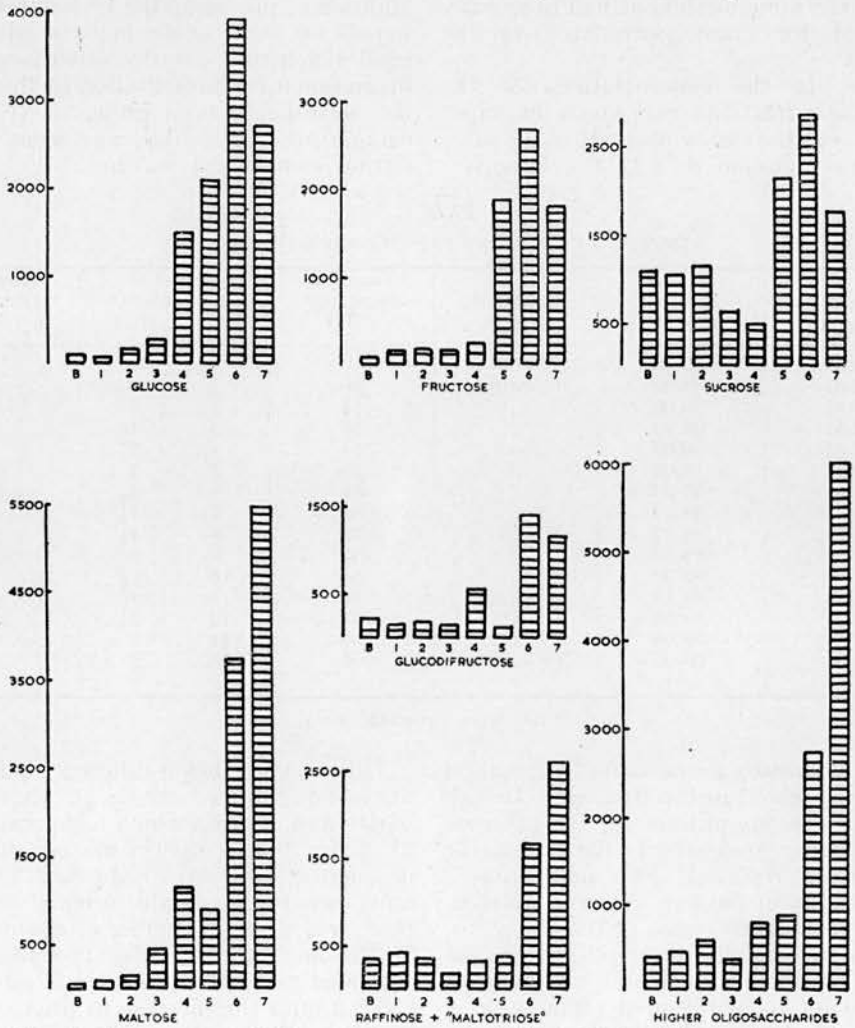

FIG. 1.—Free sugars of Ymer barley germinated at 21° C. The vertical scales represent mg. sugar per 100 g. dry matter of original grain. B = ungerminated barley; 1-7 = days of germination. For pentoses and galactose, see text.

Germination under Commercial Malting Conditions

Ymer barley from the 1951 harvest (germinative capacity 97%, moisture content 12·90%, 1,000 corn weight 41·96 g.) was selected for this part of the investigation. Samples were withdrawn from a commercial floor malting at approximately 24 hr. intervals, from the commencement of steeping till the completion of kilning. It was naturally impossible to follow the changes in a known weight of raw barley throughout all stages of malting; estimations were therefore made on 1,000 corns. At each sampling about 50 g. of barley were taken from each of three different places on the floor, the grain was mixed and 1,000 corns were counted out at random. Details of the conditions

prevailing at sampling are given in Table I. Each 1,000-corn sample was immediately treated with boiling ethanol in order to prevent, as far as possible, any further metabolic changes, and subsequent extraction and estimation of sugars was carried out by precisely the same method as had previously been used for grain germinated in the laboratory.

Changes in the concentrations of the various sugar fractions were much less rapid than had been the case when grain of the same variety was germinated at 21° C. However, a pilot experiment, designed to reveal the general trend of change in the free sugars of the grain during the first few days of germination, and if possible to give warning of difficulties to be expected in the analysis of barley germinating on the malting floor. In addition to providing the type of information hoped for, this preliminary study yielded results meriting detailed discussion, and a diagrammatic representation of these results has accordingly been presented (Fig. 1) for comparison with the analogous changes during commercial malting.

TABLE I

DETAILS OF SAMPLING FROM COMMERCIAL MALTING

Date of sampling	Hour of sampling	Origin of sample	Temp. at sampling (° F.)	Period of germination* Days	Hr.	Acrospire as fraction of corn length
1.12.51	09.00	Store	—	0	0	—
4.12.51	09.00	Steep	50	1	2	—
5.12.51	09.00	,,	51	2	2	—
6.12.51	08.30	,,	50	3	1½	—
7.12.51	09.00	Floor	54	4	2	—
8.12.51	10.00	,,	59	5	3	—
9.12.51	10.30	,,	59	6	3½	ca. ¼
10.12.51	08.30	,,	54	7	1½	¼
11.12.51	08.30	,,	53	8	1½	¼–½
12.12.51	09.30	,,	53	9	2½	ca. ½
13.12.51	09.30	,,	54	10	2½	½
14.12.51	08.45	,,	59	11	1¾	½–¾
15.12.51	10.30	,,	67	12	3½	½–¾
17.12.51	09.00	To kiln	90	14	2	¾
19.12.51	09.00	From kiln	200	16	2	—

* *i.e.*, from time into steep.

as in the laboratory germination, xylose and arabinose appeared in the free state, though not till the 6th day of malting, and raffinose rapidly disappeared from the sprouting corns, to be replaced, by "maltotriose." The estimation of the two different trisaccharides from the same region of the chromatogram was successfully accomplished by the method described above, and no evidence was found for re-synthesis of raffinose after its early depletion. In contrast to the germination carried out at a higher temperature, no free galactose could be detected during malting. The results of estimations of free sugars in barley at various stages of the malting process are shown in Table II and in Fig. 2.

DISCUSSION

Germination at 21° C. (70° F.).—The initial laboratory germination was in the nature of

The most significant difference between the disposition of free sugars in ungerminated barley and in grain which had germinated at 21° C. for 3 days was the marked diminution in sucrose content. Expressed as hexose units per 100 g. of the original dry grain, there was a loss of sucrose of 434 mg.—43% of the amount originally present. During the same period the increase in glucose was 230 mg. and the increase in fructose 53 mg. The behaviour of the other oligosaccharides containing "bound" sucrose, *viz.* raffinose and, probably, glucodifructose, resembled that of sucrose, a loss of 280 mg. being detected from the sucrose portion of these oligosaccharides by the end of the first 3 days of germination. Thus, of the total sucrose initially present, either free or forming part of raffinose or glucodifructose, some 50% was utilized during the first 3 days with no significant accumulation of fructose, and with

sufficient glucose produced to account for only two-thirds of that to be expected from the relevant loss. When it is remembered that free glucose might conceivably accumulate from hydrolysis of cell-wall material, it seems reasonable to suppose that during the early days of germination sucrose is providing, directly or indirectly, material for respiration or synthesis—or for both.

In Part II of the present study,[12] it was shown that, in a sample of Spratt-Archer barley, 44% of the raffinose and 46% of the sucrose in the ungerminated grain were located in the "3rd dust," a fraction of the grain containing the greater part of the embryo. Again, in an analysis of the sugars of barley embryos dissected free from the endosperm at various stages of germination, James[6] found that the sucrose content of the embryo fell to zero after 2 days of growth, before translocation from the endosperm had resulted in a sharp increase in free sucrose. Although a comparison involving three different varieties of barley must be couched in very general terms, these various observations would seem to suggest that, since the sucrose in the embryo may be wholly dissipated in the early metabolism of the germinating grain, the amount of this oligosaccharide in the actual germ of different barley samples might influence the nature and regularity of germination. Accumulation of data relating to the sucrose content of the embryos of different barley varieties, accompanied by detailed observations on the behaviour of these barleys during the early stages of flooring, would certainly seem to be desirable.

The appearance of galactose and of the pentoses on chromatograms prepared on the 4th and 5th days of germination coincided with a very great increase in the contents of free glucose and maltose. As has already been suggested, glucose may have come in part from hydrolysis of cell-wall material, such as the β-glucosan of Preece & Mackenzie[17]; certainly the appearance of pentoses is indicative of cell-wall degradation while the large increase in maltose suggests that amylolytic action is, at this stage of development, no longer impeded by the endospermic cell walls. Free galactose could have originated from hydrolysis of various different materials, including raffinose and water-soluble gums, but as the fraction including raffinose was not fully characterized in this first part of the investigation, discussion of the behaviour of raffinose and of galactose will be deferred until the results of the analysis of barley during malting are considered.

As has already been indicated, the fructose content of the grain showed little change during the first 4 days of germination

TABLE II

FREE SUGARS OF BARLEY (YMER) DURING MALTING

Origin of sample	Period of germination Days	Hr.	Mg. of sugar per 1,000 corns							
			Glucose	Fructose	Maltose	Sucrose	Glucodi-fructose	Raff-inose	"Malto-triose"	*Higher oligosaccha-rides
Store	0	0	34	41	22	421	92	203	—	326
Steep	1	2	29	40	35	304	40	114	—	226
,,	2	2	24	49	28	306	43	50	12	90
,,	3	1½	22	45	36	426	123	37	42	69
Floor	4	2	22	44	31	246	43	14	44	32
,,	5	3	64	59	93	355	81	9	47	51
,,	6	3½	91	98	175	599	108	8	54	118
,,	7	1½	355	126	337	809	147	—	110	263
,,	8	1½	316	140	365	532	189	—	200	369
,,	9	2½	661	112	494	867	265	—	232	146
,,	10	2½	792	283	813	1,504	457	—	312	735
,,	11	1¾	922	266	744	1,597	468	—	369	634
,,	12	3½	973	255	800	1,366	243	—	147	338
To kiln	14	2	1,021	214	789	1,936	318	—	180	355
Kiln†	16	2	629	221	299	2,100	180	—	74	181

* Soluble in 90% ethanol.

† Finished malt (with rootlets).

although sucrose was gradually diminishing. On the 5th day, however, fructose increased ninefold, simultaneously with a marked rise in free sucrose—the first since the inception of growth. This accumulation of free fructose could result from inversion of sucrose or from partial or complete hydrolysis of glucodifructose or other fructose-containing oligosaccharides of high molecular weight. Until a fuller analysis of the fructosans is available, little would be gained from speculation on the origins of the free fructose of the germinating grain.

The principal changes observed in the concentration of the different sugars in barley germinating at 21° C. may conveniently be summarized in the following way: The concentrations of all sugars and oligosaccharides containing a "sucrose" grouping decreased over the first 4 days, without any marked simultaneous increase in fructose. After the 4th day the concentrations of fructose and sucrose rose steeply. In contrast to the sugars containing a fructofuranose unit, the pyranose sugars, glucose and maltose, showed a gradual increase, these pyranose sugars probably being derived principally from starch hydrolysis. The products of dissolution of the cell walls of the endosperm—xylose and arabinose, together with a proportion of the glucose and, possibly, galactose—appeared in minor quantities in the 4th day of germination, but the pentoses did not appear to accumulate, and, indeed, could not be detected with certainty in grain which had been germinated for 6 days.

Commercial malting.—With the grain germinating under commercial malting conditions, the course of events was in general rather similar to that observed at 21° C., though the lower temperature prevailing throughout (with, consequently, a slower rate of metabolism) made possible a larger number of observations of the same total change.

Once again, after 4 days, *i.e.*, 72 hr. steeping and 24 hr. on the floor, the diminution in sugars and oligosaccharides containing a sucrose group was most striking. In the ungerminated grain, the total content of hexoses combined as sucrose (*i.e.*, the sucrose content of raffinose and glucodifructose, in addition to free sucrose) amounted to 710 mg. per 1,000 corns. After 4 days of germination, the comparable figure was 290 mg.—a loss of nearly 60%. The disappearance of raffinose was most marked, only the merest traces of that trisaccharide being detectable after 4 days. During the period of diminution of sucrose there was no significant change in either the glucose or the fructose content of the germinating grain. Clearly, then, the sucrose components do not accumulate as monosaccharides. It is significant, too, that concurrently with the fall in sucrose and raffinose, there was a steady fall in the concentrations of the higher oligosaccharides originally present in the grain. In ungerminated barley these oligosaccharides appear to constitute an homologous series based on sucrose with individual members containing increasing numbers of fructose units.[13] In artichoke tubers, a similar series of fructosans can participate in transfructosidations which may involve transfer of anhydrofructose units to various members of the series[4]; the incorporation of sucrose in glucodifructose, or in other fructosans, could thus account for the disappearance of sucrose without accumulation of the constituent monosaccharides. It would appear, however, that such transfructosidations, if they occur at all, are not of great magnitude in the early days of barley germination as there is no suggestion of any increase in any fructose-containing fraction. Indeed, the fructosans in the ungerminated grain show a diminution in quantity similar to that observed for other oligosaccharides containing fructofuranosidic units (higher oligosaccharides, Fig. 2).

It is interesting to note that, despite the rapid utilization of raffinose, no clear evidence was secured for the occurrence of free galactose at any stage of the malting process. It is possible that traces of melibiose, derived from raffinose, may have been included in the glucodifructose fraction, as melibiose and glucodifructose occupy the same area of the paper on chromatograms partitioned in butanol-acetic acid-water. The spot in the glucodifructose region was not examined critically at all stages, but a chromatogram prepared on the 9th day of germination gave a strong ketose reaction and completely failed to indicate any trace of reducing sugar in the glucodifructose region. The former reaction suggests the continued presence of a low-molecular fructosan, while the latter definitely points to the absence of melibiose. The fate of the galactose unit of raffinose must therefore remain at present uncertain; it does not

accumulate in the free state, and both the actual figures for sugar content in the melibiose region and the results of differential spraying suggest that it cannot wholly be accounted for as melibiose.

detected before the 5th day of germination. Thereafter a steady increase in glucose, in maltose and in the freshly formed "maltotriose" continued up till the 10th day, when, with the gradual withering of the grain,

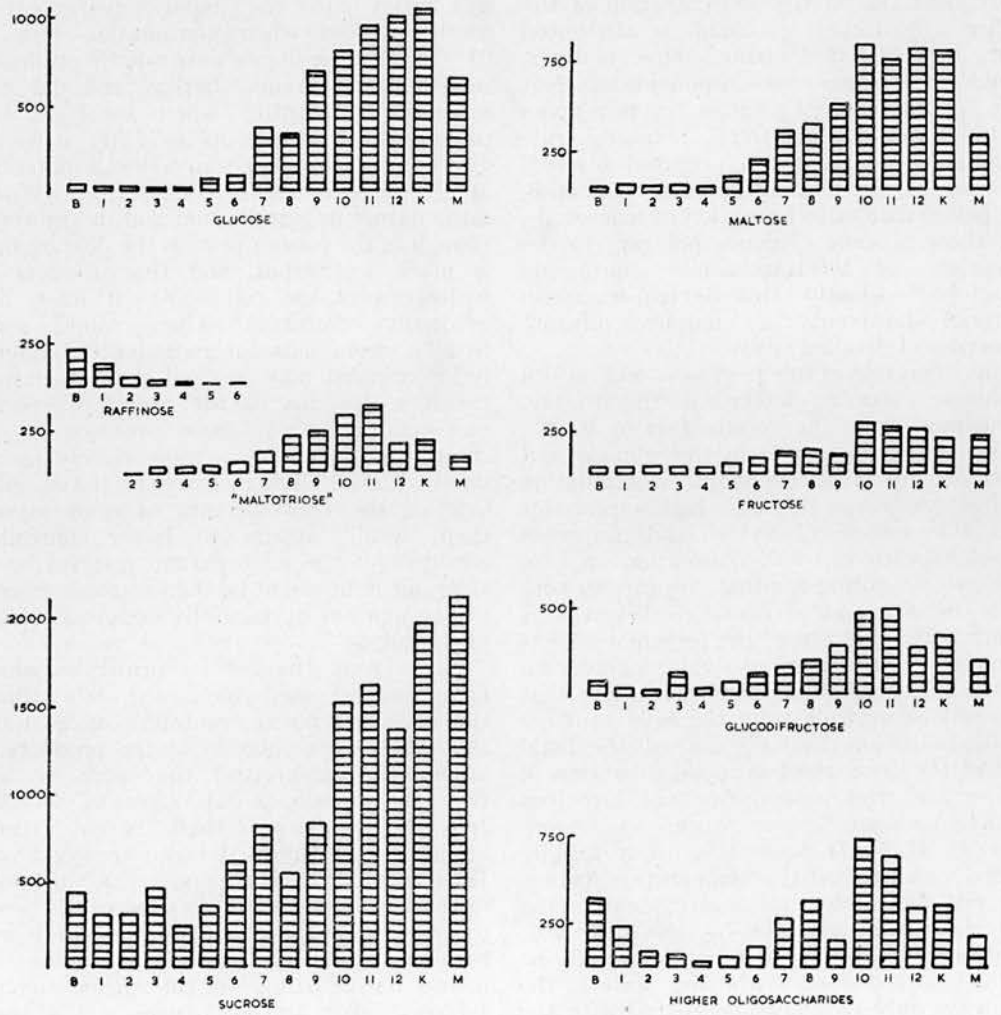

FIG. 2.—Free sugars of Ymer barley during malting. The vertical scales represent mg. sugar per 1,000 corns. B = ungerminated barley; 1-3 = days in steep; 4-12 = days on floor; K = commencement of kilning; M = kilned malt with rootlets. For pentoses, see text.

The accumulation of the sugars and oligosaccharides related to starch, *viz.*, "maltotriose," maltose and glucose was remarkably regular throughout the whole malting process. Glucose and maltose were both present in low concentrations in the ungerminated grain and no marked increase was

"maltotriose" decreased, maltose remained constant, and glucose showed a slight rise. If "maltotriose" is to be accounted for by amylolysis in the germinating grain, it is to be expected that maltotetraose, maltopentaose, etc., would also occur. These oligosaccharides would form part of the higher

oligosaccharide fraction, which has not yet been analysed in detail. By analogy with the behaviour of the trisaccharides, it might be concluded that the fructosans of the original grain would be exhausted by the 5th and 6th day of germination and that the subsequent rise in the concentration of the higher oligosaccharides could be attributed to the formation of dextrins. This, however, would be a gross over-simplification. Not only is a characteristic ketose reaction given by the material in the tetra—octasaccharide region of chromatograms prepared towards the end of the malting process, indicating the persistance or re-formation of fructosans, but there is some evidence pointing to the formation of tetrasaccharides containing galactose.[18] Clearly, this dextrin-fructosan material represents a complex mixture deserving of detailed study.

The liberation of free pentoses—xylose and arabinose—was first detected on the 9th day, coinciding, as in the germination at 21° C., with a marked increase in free glucose and preceding by 24 hr. the major accumulation of free fructose. It seems highly probable that this release of xylose and arabinose coincides with cell-wall dissolution—a process, which, during malting, appears to continue till the end of flooring. When it is remembered that one of the principal objects of malting is to secure adequate degradation of the cell walls of the endosperm, and that this process spreads from the cells abutting on the scutellum gradually towards the distal end of the corn, the continued liberation of xylose and arabinose during the last four days on the floor is not surprising. Germination at 21° C. is equivalent to a rapidly forced malting. At this temperature, pentosan cell wall dissolution was evident in the 4th day and complete by the 6th day; pentoses were produced twice as rapidly at 21° C. and remained in the free state in the grain for only two days, compared with the four days of persistance during commercial malting. It would be inadvisable at present to speculate on the fate of the liberated pentoses, for, though knowledge of the structure of the hemicelluloses of the cell wall is gradually accumulating, virtually nothing is known of the means by which pentosans are synthesized.

Concurrently with this study of the sugars of germinating barley, analyses were made of the accumulation of free sugars in autolysing grain. Although the results of this work on autolysis are not of sufficient relevance to quote here in full, it may be noted that the increase in pyranose sugars during germination bore a strong resemblance to the autolytic accumulation of maltose and glucose in a barley mash, the similarity being particularly marked when germination was at 21° C. Pentose sugars were rapidly produced in autolysing ground barley, and did not increase in quantity when autolysis was prolonged for periods up to 72 hr.; it would appear that the changes in cell wall materials and in the starch of the endosperm are of the same nature in germination and in autolysis, though in the former process the degradation is much less rapid, and the products of hydrolysis of the cell walls, at least, are gradually re-utilized. There would seem to be a strong case for assigning the amylolytic enzymes, maltase, and the cytoclastic-cytolytic systems to the group of special metabolic enzymes whose presence is ancillary to the processes more closely associated with the "living" activities in the cell.[11] One of the concomitants of germination, then, would appear to be a controlled autolysis of the endospermic reserves; considerable light might be shed on some aspects of germination by carefully designed studies on autolysis.

Apart from the general principles which have been discussed above—the early utilization of glucopyranose-fructofuranosidic units, the steady accumulation of the products of amylolytic action and the more or less transient appearance of products of pentosan cell-wall degradation—there are few features at present worthy of detailed consideration. It is reasonable to suppose that variations in the temperature and aeration of the piece, together with the effects of sprinkling and turning, would exercise a considerable influence on the balance of the various enzyme systems active during malting. The transient accumulation of sucrose towards the end of steeping, the fall in sucrose content balanced by a rise in fructosans on the 8th day, are obvious interruptions in the smooth series of changes but, doubtless, had a different floor been examined, deviations from the general trend of events would have been observed at different stages in the process. The resemblances between the sugar metabolism in a floor malting and a laboratory germination at 21° C. are at present, however,

of greater significance than minor irregularities, though it is possible that, with a fuller understanding of the changes usually associated with successful malting, recognition of departures from the normal might provide information of value to the maltster.

Kilning.—In the final analysis of the kilned malt, marked changes were noted in the concentrations of the free sugars. Thus, free pentoses could no longer be detected, and glucose, maltose and "maltotriose" showed marked diminution; sucrose, and to a very minor extent, fructose, continued to increase. The loss of pentoses and the disappearance of part of the free glucose may readily be attributed to the formation of condensation compounds with amino acids; the rise in sucrose is less easily accounted for, though the losses of glucodifructose and higher oligosaccharides are more than sufficient to account for the extra sucrose produced. The increasing temperatures during kilning may have resulted in an alteration in the equilibrium of the sucrose-fructosan group of oligosaccharides, differential effects on the various enzymes having mediated the synthesis or hydrolysis of this series of compounds. This increase in sucrose content after kilning has frequently been noted,[3] though there is not as yet agreement as to its origin.

A comparison of the free sugars of ungerminated grain with those of kilned malt is given in Table III. It must be emphasized that the results presented here refer to one sample of Ymer barley, which was steeped, floored and kilned under specific conditions; it is improbable that these figures are of universal application to all varieties of barley or to different malting conditions, though there is little doubt that the same general principles will apply.

Comparison with previous work.—As the methods used in the present study are relatively new, it would be desirable to consider the results in conjunction with similar data previously obtained by more orthodox methods. It was shown in Part II of this investigation[12] that the figures obtained by chromatographic separation and micro-estimation were in general agreement with results previously recorded for ungerminated grain, some of the discrepancies being attributable to the presence in barley of previously unrecognized oligosaccharides. Although the results here obtained for the free sugar content of malt (Table III) are of the same order as some of those previously reported, it is rather more difficult to achieve valid comparisons at the intervening stages of germination, as the various chemical analyses of barley during malting show marked discrepancies one with another. Thus, until recently, the presence of maltose in barley and in malt was a matter for discussion. O'Sullivan[15] recorded the presence of this sugar in malt, but Ling[9] failed to detect it and de Clerck[2] asserted that free maltose was never found in barley or in malt, since it was immediately hydrolysed by maltase; Kluyver[8] reported the presence of maltose in grain which had germinated for 3 days, and James[6] detected free maltose in barley embryos. The results of chromatographic analyses of extracts prepared from material in which enzymes had been completely inactivated have now made it abundantly clear that maltose is a normal constituent both of barley and of malt, though the actual amount of maltose present

TABLE III

FREE SUGARS OF AN YMER BARLEY AND OF ITS MALT

	Mg. of sugar per 100 g. of dry matter							
	Glucose	Fructose	Maltose	Sucrose	Glucodi-fructose	Raffinose	"Malto-triose"	*Higher oligo-saccha-rides
Barley	92	111	59	1,137	248	548	—	880
Malt†	1,855	625	882	6,095	531	—	218	534

* Soluble in 90% ethanol.

† With rootlets.

in barley may vary over a very wide range from sample to sample.

Even when allowance is made for possible varietal, cultural and storage differences, it is clear that, where there is no agreement as to the presence or absence of a particular sugar, comparisons of concentration data can be only of limited value. Brief reference may be made, however, to two analyses of germinating barley, for very general comparative purposes. Lüers & Loibl[10] estimated the free sugars as "invert" and "sucrose" throughout the entire malting process, and, in so far as comparison is possible, their results appear to be in general agreement with those quoted in the present investigation. Thus, invert remained virtually unaltered in concentration during steeping, diminished appreciably during the first two days of flooring, and showed a sudden major increase on the 4th day so that the final concentration at the end of flooring was about nine times that in the original barley. Sucrose content fell during steeping and, as in the present investigation, showed a fluctuating rise after the 2nd day on the floor till, at the end of 7 days of growth, the concentration of sucrose was some two and a half times as great as that of ungerminated grain. Kluyver,[8] who made a more detailed examination of the sugars, found that raffinose had vanished from the grain after 24 hr. of flooring, that maltose and the hexoses showed a simultaneous five-fold increase on the 4th day, and that sucrose, after diminution in the steep, increased in concentration, fell slightly then increased again to reach a level of 4% of the dry weight after 7 days.

Examination of the histograms of Fig. 2 shows that the general trends of change in sugar concentrations described in the present investigation are similar in nature to those established by these purely chemical methods; since the results for ungerminated barley and for malt are also in reasonable agreement with previous figures, it would seem that the chromatographic method, if carefully applied, is at least as reliable as the more laborious chemical methods. Chromatography, moreover, has the great merit of discriminating between oligosaccharides of similar chemical nature but of different molecular sizes.

It must be stressed, however, that very great caution should be applied in interpreting the results of paper partition chromatography, especially when the higher oligosaccharides are involved. Sugars which have identical R_F values in several different solvents are not necessarily structurally identical; the replacement of raffinose by "maltotriose" in germinating barley and, quite literally, on the chromatograms prepared from that barley, amply illustrates this point. In this connection, some doubt must be cast on the identification by Montreuil & Scriban[14] of raffinose in malt; several earlier analyses have shown that raffinose rapidly disappears from germinating grain, and results of the present study suggest that the sugar in question is not raffinose but some trisaccharide possibly arising from starch degradation.

Conclusions

It was stated in the introductory paragraphs that analysis of sugar concentrations in germinating barley can be expected to reveal little more than the balance in the grain at the time of analysis. Results of the present study, however, have indicated a number of definite trends in the accumulation or utilization of certain specific sugars and oligosaccharides—trends which certainly merit more detailed investigation. Thus, analogies between uncontrolled autolysis and certain aspects of the accumulation of pyranose sugars have been suggested, the general similarity of behaviour of all oligosaccharides containing glucopyranose-fructofuranosidic groups has been noted, and the disappearance of raffinose at an early stage in germination has been confirmed. To what extent can these trends be linked with the threefold equilibrium of hydrolysis, respiration and synthesis?

Rapid mobilization of endospermic reserves may account for the temporary accumulation of pentoses, maltose and glucose; sucrose depletion has, on occasion, been correlated with respiration in the embryo,[7] but the details of synthesis of new cellular material remain obscure, though hemicellulose-like substances may increase fivefold in the embryo during the first six days of germination at 21° C.[6] Any carbohydrates intermediate in complexity between the free pentoses and the water-soluble gums associated with the cell wall would be expected to appear in the heterogeneous group of substances designated "higher oligosaccharides," and, though there is no definite evidence for or against the occurrence of such intermediates, a full

analysis of the higher oligosaccharides of germinating barley might yield information of considerable interest.

Three specialized features of the carbohydrate metabolism of germinating barley have therefore been selected for more detailed study; the relationship of germination to autolysis, the peculiar status of raffinose and sucrose, and the identity and origin of the higher oligosaccharides. It is hoped that the results of work now in progress on these specific problems may add to the present knowledge of the carbohydrate metabolism of barley during malting.

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IV

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN

IV. LOW-MOLECULAR FRUCTOSANS

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IV. LOW-MOLECULAR FRUCTOSANS

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Ungerminated barley yields a series of fructose-containing oligosaccharides which can be separated, on charcoal-kieselguhr columns and by paper chromatography, into at least six separate fractions. The simplest member of the fructosan series is glucodifructose, with smaller quantities of glucotrifructose, glucotetrafructose and glucopentafructose also present; the more complex fructosan material, ranging up to glucodecafructose, has not as yet been fractionated into discrete compounds. In addition to easily-hydrolysable fructosan, one sample of Ymer barley contained traces of an oligosaccharide, in the tetrasaccharide region of the chromatogram, which was only partially hydrolysed by invertase or by acetic acid.

Introduction

In the work previously reported in this series,[17,18,19] attention has been directed mainly towards the simpler sugars and oligosaccharides of the barley grain, and some knowledge of their identities,[16] their ranges of concentration in different samples of barley,[18] and their fluctuations during malting[19] has gradually been accumulated. At an early stage in this investigation, however, it was realized that in addition to the relatively familiar plant sugars, glucose, fructose, sucrose, raffinose and maltose, there existed in barley a rather ill-defined series of non-reducing oligosaccharides, which, from their chromatographic behaviour, seemed to range from a trisaccharide to oligosaccharides containing upwards of seven hexose units. The principal hydrolysis product of this material was fructose, with smaller amounts of glucose also apparently present, and the term "fructosan" has been applied generally to this series of compounds. In the earlier work, a proportion of this material was inevitably extracted, in order to secure complete separation of all sugars, and, though little was known of its constitution, some account has already been given of the behaviour of the lower-molecular fructosans during malting.[19] Similarly, glucodifructose which, as will be shown later, is most appropriately considered along with the fructosans, has already been estimated among the simpler sugars, and its behaviour during malting has been fully studied. Apart from these preliminary observations, little work has as yet been reported on the low-molecular fructosans of barley.

Desultory interest, however, has been shown in the high polymers of fructose for very many years. Thus Kuhnemann,[14] in 1875, deduced the presence in barley of a fructosan type of material and assigned to it the name sinistrin; some 10 years later Muntz[20] prepared from the same source an amorphous substance which was largely fructosan, and Tanret,[22] in 1891, showed that apparently similar material (levosine) had a specific rotation of −43°. A quarter of a century later, Colin & Belval[8] and de Cugnac[9] carried out extensive investigations of the fructosans of barley and other cereals and grasses, and concluded that fructosans from different sources differed in such properties as water-solubility, alcohol-solubility, and susceptibility to hydrolysis by invertase. Purification of fructosans has, in general, been carried out by ethanolic precipitation combined with baryta treatment, and, in this early work, opinion was divided as to the constitution of the purified products. Thus, with certain preparations, the fructose appearing after hydrolysis was accompanied by small quantities of glucose, and final decision could not be made as to the origin of this glucose; it might represent an integral part of the fructosan, it might have arisen from slight contamination with sucrose, or it might have been derived from a glucosan with precipitation properties similar to those of the fructosan.

Recently, methods involving methylation

and chromatographic separation and identification of the products of hydrolysis of the methylated polysaccharide have been applied to fructosans isolated from leaves of various grasses, and fructosans from *Dactylis glomerata*,[7] *Triticum repens*[4] and *Lolium perenne*[15] have all been shown to contain approximately 5% of glucose; the position of the glucose residue is at the end of the main fructose chain and the linkage is such that the fructosan terminates in a sucrose residue. Even the most familiar of all fructosans, inulin, has recently been shown to contain 1 glucose unit to approximately every 35 fructose units,[7,13] and, though the types of linkage in the main chains of fructosans from different sources may differ, all so far examined resemble one another in containing this terminal sucrose unit.

The work of these authors, however, has been concerned mainly with fructosans containing 20 or more hexose units; the material observed on chromatograms of barley extracts would appear to range down to a lower limit of 3 hexose units. That little work has been carried out with respect to the low-molecular fructosans of barley is not surprising, as even the recognition of these compounds was difficult before the introduction of partition chromatography. Archbold,[2] however, from a consideration of the results of the action of invertase on an extract of barley stems, deduced the presence of fructosan material, more susceptible to invertase than the well-known high-molecular fructosan, and containing a certain proportion of aldose sugar in the molecule. Again, Bacon & Edelman[6] have recently shown that the Jerusalem artichoke and certain other members of the Compositae contain a series of fructose-containing oligosaccharides in addition to inulin, the high-molecular polymer of fructose; in common with inulin, the individual members of this series were each terminated by a glucose unit. Studies of oligosaccharides from the endosperm of wheat[25] have revealed the presence there of a range of fructose-containing compounds, a total of five substances of lesser chromatographic mobility than raffinose being detected after prolonged partition in butanol-ethanol-water. Scattered observations made throughout the present investigation[18,19] suggest that barley resembles other plant materials known to contain high-molecular fructosan in yielding a range of compounds intermediate in complexity between sucrose and the high-polymer material. A more detailed study has now been made of these fructose-containing oligosaccharides, and the results of that study are reported in this communication.

Experimental

Fractionation methods. — Enzyme-inactivated barley, prepared in the usual manner,[18] was finely-ground and extracted with water at room temperature for three successive 1-hr. periods. The extracts were bulked and concentrated to 50 ml. in presence of a trace of barium carbonate, an equal volume of 50% ethanol was added and the resulting precipitate (principally water-soluble gums) was removed by centrifugation. The clear solution was further concentrated to *ca.* 5 ml., prior to chromatographic separation. Aqueous extraction was used for preparation of fructosans rather than the usual ethanolic extraction, as the high-molecular material is only sparingly soluble in 80–90% ethanol, but completely soluble in 50% ethanol. Attempts were initially made to separate the fructosans on Whatman No. 1 paper by the methods previously described,[18] but the amounts of the individual fractions recovered proved insufficient for detailed analyses; the large quantities of sucrose present in the extracts, and the low absorptive capacity of this grade of paper, together formed a mechanical barrier to the separation of more than a fraction of a mg. of each component of the high-molecular material. Whatman No. 4 paper, which gave much more rapid separation of sugars and oligosaccharides, had the same disadvantage as No. 1, but Whatman 3MM, a thick paper which permitted rather more rapid separation than No. 1, proved reasonably satisfactory; after 5–7 days of partition a number of dark, more or less discrete bands extending from raffinose to the starting line of the chromatogram could be detected by spraying with aniline oxalate. Of the different solvent systems investigated, mixed butanol-acetic acid-water (40 : 10 : 50) proved to be the most generally useful; development with this solvent was slower than with butanol-benzene-pyridine-water or with ethyl acetate-*n*-propanol-water, but separation of the higher oligosaccharides was more clear-cut.

Although paper partition permitted fairly complete characterization of fructose-containing trisaccharides, and yielded quantities

of the less mobile fructosans sufficient to allow identification of their component units, the amounts recovered were still inadequate for precise quantitative determinations. An attempt was therefore made to separate the higher fructosans on a charcoal-kieselguhr column.[24] To prepare such a column, B.D.H. decolorizing charcoal was well washed (to pH 7) and mixed into a slurry with an equal weight of kieselguhr; the slurry, which was poured into a glass tube 5·5 cm. in diameter, was then allowed to settle to form a compact homogeneous mass 9 cm. high. After excess water had drained from the column, the barley extract was introduced and washed down with 25 ml. of water; the column was then connected by means of a siphon to a reservoir containing initially 5% ethanol and later increasing concentrations up to 30% ethanol. The effluent from the column was collected, without suction, and each separate ethanolic fraction was concentrated to *ca.* 5 ml. and examined by the usual papyrographic method. Results of such a separation are given in Table I. It was found that 7½% ethanol removed all monosaccharides and disaccharides together with some of the trisaccharides, and that increasing concentrations of ethanol were increasingly effective in eluting higher oligosaccharides. About 1,500 ml. of each concentration of ethanol were allowed to percolate through the column; the first 200 ml. of each percolate was examined separately, since residual material—not completely desorbed by the preceding concentration of ethanol—was rapidly eluted by the higher concentration.

In view of the success of this preliminary fractionation, attempts were made to obtain complete separation of individual fructosans, directly from the column. To this end, 20-ml. portions of the various ethanolic percolates were collected by means of an automatic fraction-collector, and the contents of every fifth tube were concentrated and examined chromatographically. Results, however, were disappointing. Too little material was present in the 20-ml. aliquots to be of any value for detailed analysis, and when several consecutive aliquots were bulked, chromatographic examination revealed that more than one oligosaccharide was present. Columns were therefore used mainly to give preliminary separation of the higher oligosaccharides from sucrose and the monosaccharides; as these sugars frequently amounted to 50% of the total carbohydrates in the final extracts, this represented a substantial purification of fructosan material, and rendered the final solution much more amenable to papyrographic separation. For routine work, therefore, the column containing the barley extract was washed with 1,500 ml. of 7½% ethanol, which was discarded, and elution was then carried out with 30% ethanol. This ethanolic solution was concentrated in presence of a trace of barium carbonate and further separated on 3MM paper. The presence of barium carbonate was particularly important at this

TABLE I

FRACTIONATION OF ELUATE FROM CHARCOAL-KIESELGUHR COLUMN

Concentration of ethanol applied to column (%)	Volume of eluate collected (ml.)	Oligosaccharides present on chromatogram	Sugars in hydrolysate (mg.)		Ratio of glucose to fructose (G : F)
			Glucose	Fructose	
7½	1,400	Sucrose, maltose; trace of glucodifructose	—	—	—
10	1,550	Glucodifructose, raffinose; trace of spot above raffinose	—	—	—
12½	1,100	Double spot above raffinose insufficient for hydrolysis	—	—	—
15	1,100	Fructosan	0·1621	0·5912	1 : 3·6
17½	1,000	,,	0·1911	0·8689	1 : 4·5
20	950	,,	0·1041	0·5763	1 : 5·5
22½	1,000	,,	0·1431	0·8801	1 : 6·0
25	850	,,	0·1502	1·0602	1 : 7·0
27½	650	,,	0·0751	0·5456	1 : 7·2

stage, when the solution was virtually free from buffering agents; in the absence of barium carbonate "autohydrolysis" (*cf.* Aspinall & Telfer[5]) occurred, and partially-degraded fructosan, together with fructose and a substance with the same R_F value as sucrose, appeared in the concentrate.

Examination of separated fractions.—Each fraction under examination was washed off the paper, an aliquot was boiled under reflux for 15 min. with 50% acetic acid and, after concentration, the products of hydrolysis were identified chromatographically. A second aliquot was treated with 2% B.D.H. invertase concentrate for 24 hr. at 37° C. and pH 4·6; the products of inversion were also identified chromatographically. Complete hydrolysis of the majority of the fractions examined was achieved by either of the above methods, but in the absence of buffer (*i.e.*, at pH 6·8) the fructosans were virtually unaffected by invertase. The fraction which did not leave the starting line of paper chromatograms was only partially hydrolysed by invertase, even after 48 hr.; with acid, however, hydrolysis was complete.

In order to determine the relative amounts of the constituent sugars of the different fructosans, the appropriate strips of paper from the chromatographic separation of the hydrolysate were eluted in the usual manner and, in the first instance, sugars were estimated by means of the Somogyi micro-copper reagent.[21] The quantities of sugar concerned were, however, rather small for accurate titration differences, especially in the case of the less mobile fractions, and use of a method suited to determination of μg. quantities of sugar was therefore considered desirable. The anthrone reagent, which has successfully been used for determining yeast carbohydrates,[23] was investigated and appeared to give adequate readings with the quantities of monosaccharides recoverable from the chromatograms. For standardization of the reagent, sucrose was treated for 24 hr. with invertase and the resulting glucose and fructose were separated on paper, eluted, and made to known volumes (usually about 25 ml.) before treatment for varying lengths of time and in varying concentrations with anthrone. These standardization conditions were chosen to resemble as closely as possible those under which subsequent estimation of unknown quantities of sugar would be carried out. The reagent was prepared daily by dissolving 0·2 g. of anthrone in 100 ml. of concentrated sulphuric acid, and 6 ml. of this reagent were added to 3 ml. of sugar solution, rise of temperature being minimized by rotating the reaction tubes in a bath of ice-cold water while the sugar solution and the reagent were being mixed. Despite this careful cooling, the heat engendered during the reaction with fructose was sufficient to cause substantial colour development, and, though this was an undesirable feature in view of the need for precise timing, it gave

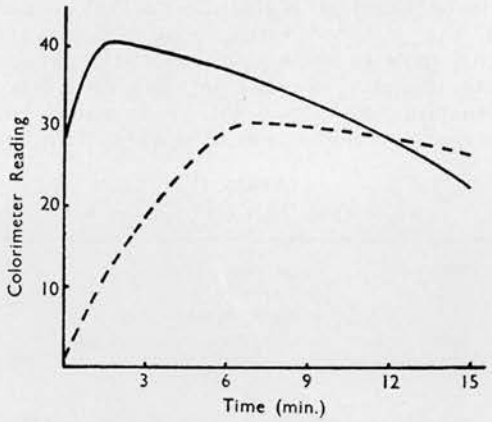

Fig. 1—Anthrone colour development with fructose (full curve) and with glucose (broken curve).

a useful indication of any slight contamination of glucose by fructose. After mixing, the tubes were heated for a precise known time in a boiling water bath, and rapidly cooled in ice-cold water. The light transmission of the green solution was then determined by means of an EEL colorimeter using No. 607 filter, and compared with a blank in which washings from a portion of the chromatogram free from sugar were used in place of the sugar solution. Curves of colour development against time of heating are shown in Fig. 1.

Maximum colour intensity was reached in the solutions containing fructose after only 1½ min. heating, whereas the maximum value for glucose was not attained until heating had been continued for 6–8 min. After precisely 12 min. in the boiling water bath, under the conditions described, equal quantities of glucose and fructose showed identical colour intensities; this length of time was therefore chosen for the subsequent determinations. Linearity was observed by both glucose and fructose between colorimeter readings of 6° and 60°.

In carrying out determinations with unknown quantities of sugars, eluted material was first made to an arbitrary volume, and 1 ml. was used to ascertain the approximate sugar concentration; after appropriate dilution, the remainder was used for precise determinations. Experience of the relative intensities of the spots on the indicator strips cut from the margin of the chromatogram proved helpful in deciding the probable extent of dilution required.

From a consideration of the shape of the curves (Fig. 1), it is clear that a slight degree of over- or under-heating in the boiling water bath may cause a significant error in the ratio of glucose to fructose. Replicate determinations were therefore not made concurrently in the same boiling water bath, but as separate successive estimations, and the mean of three such estimations was considered the minimum for acceptable accuracy.

TABLE II

HYDROLYSIS OF GLUCODIFRUCTOSE

Agent of hydrolysis	Sugar recovered from chromatogram of hydrolysate (mg.)		Ratio of glucose to fructose G:F
	Glucose	Fructose	
Acetic acid	0·133	0·276	1:2·07
,, ,,	0·488*	0·930*	1:1·91
,, ,,	0·220	0·438	1:1·98
,, ,,	0·191	0·399	1:2·09
Invertase ..	0·256	0·461	1:1·80
,, ..	0·169	0·322	1:1·90
,, ..	0·176	0·322	1:1·83
,, ..	0·162	0·315	1:1·94
,, ..	0·380*	0·790*	1:2·08
,, ..	0·790*	1·570*	1:1·99
Totals ..	2·965	5·823	1:1·96

* Glucodifructose initially separated on charcoal-kieselguhr column.

Results.—It has previously been shown that the oligosaccharide which occupies a position immediately below that of raffinose on papers developed in butanol-acetic acid-water consists wholly of glucose and fructose.[16] The chromatographic mobility of this oligosaccharide suggests that it is a trisaccharide and its rapid complete hydrolysis by invertase indicated that it might be a glucosido-fructosido-fructoside. In the course of this investigation, many samples of this presumed trisaccharide were separated from the adjacent sugars, and results of determinations of its hydrolysis products, carried out by the methods described above, are shown in Table II. It is clear that this oligosaccharide is indeed a glucodifructose, and, in view of the ease of hydrolysis by invertase, it presumably contains β-fructosidic linkages.

In preparing hydrolysates of glucodifructose, occasional instances were encountered where there had apparently been slight contamination by raffinose, which occupies a position immediately above glucodifructose in the initial separation. Such contamination was readily detected by the presence of melibiose amongst the hydrolysis products, and any such contaminated chromatograms were rejected. In analysing the oligosaccharide fraction which appeared above raffinose, it was observed, with one sample of barley, that in addition to glucose and fructose, partially-hydrolysed material appeared in the position normally occupied by melibiose. At first this was attributed to contamination with raffinose from the original separation, but the regularity with which such apparent contamination occurred despite careful cutting of the paper, and the fact that raffinose could not be detected on chromatograms of eluted unhydrolysed material, suggested that an oligosaccharide was present which was not susceptible to complete hydrolysis by invertase. The amounts concerned were small, only one sample (of Ymer barley) exhibited this

TABLE III

ANTHRONE ESTIMATION OF HYDRLOYSIS PRODUCTS OF FRUCTOSANS

Serial fractions from glucodifructose to origin	Ratio of glucose to fructose in hydrolysis products (G:F)
Glucodifructose	1:2
Raffinose..	—
1*	1:3·9
2	1:4·6
3	1:5·0
4	1:5·6
5	1:5·9
6	1:6·4
7 (starting line) ..	1:9·9†

* Each number represents the eluate from successive 1-cm. strips of paper.

† Trace of unhydrolysed material left at starting line.

TABLE IV

ANALYSIS OF INDIVIDUAL FRUCTOSANS

Position of spot on chromatogram (butanol-acetic acid-water)	Products of hydrolysis (invertase)	Sugar recovered from chromatogram (mg.)		Ratio of glucose to fructose (G : F)	Probable composition
		Glucose	Fructose		
Below raffinose	Glucose and fructose	0·162	0·315	1 : 1·94	Glucodifructose
Opposite to raffinose	Melibiose and fructose	—	—	—	Raffinose
1st above raffinose	Glucose and fructose	0·218	0·714	1 : 2·88	Glucotrifructose
2nd above raffinose	,, ,, ,,	0·0438	0·178	1 : 4·06	Glucotetrafructose
3rd above raffinose	,, ,, ,,	0·160	0·786	1 : 4·91	Glucopentafructose
4th and 5th above raffinose	,, ,, ,,	Insufficient for estimation		—	—
On starting line	,, ,, ,,	0·146	1·523	1 : 10·4*	Mixture of higher fructosans

* Trace of unhydrolysed material left on starting line.

anomaly, and no further investigation has as yet been made of this difficultly-hydrolysable oligosaccharide.

With this one exception, all fractions of the fructosan material which showed any mobility in butanol-acetic acid-water were completely hydrolysed, either by invertase or by acetic acid, into glucose and fructose. As can be seen from Table I, which summarizes the results of a typical charcoal-kieselguhr separation, increasing proportions of fructose were present in the hydrolysates of material recovered in the increasingly higher concentrations of ethanol, and in all cases a greater or lesser quantity of glucose was detected. With the material which required 27½% ethanol for elution the values for the Somogyi estimation of glucose were too low for accurate determination even of proportionate values, as the figure recorded (0·0751) represents a titre difference of only 0·5 ml.; the ratio of glucose to fructose (1 : 7·2) must therefore represent only an approximation. Similar results were obtained, however, when all the fructose-containing material of greater molecular complexity than raffinose was divided into seven crude fractions, by cutting strips from a chromatogram, for analysis by the anthrone method after the usual inversion and re-partition. Typical results are given in Table III. Each ratio is derived from at least three separate EEL readings, and all values were well within the limits of linearity.

It is clear, from the results quoted in Tables I and III, that the different fractions analysed did not in general represent individual separated oligosaccharides, but rather mixtures of closely-related fructosans; indeed, the highest members of the series, which did not leave the starting-line of the chromatogram, may have contained a whole range of oligosaccharides of different molecular complexities. The ratio of 1 part of glucose to 10 parts of fructose—the highest proportion of fructose detected—may thus represent a spread of from 1 : 6 to 1 : 14 or more, but repeated attempted fractionation on columns with concentrations of ethanol ranging from 30–40% did not meet with success, the amounts of oligosaccharide in each fraction proving too small for hydrolyses and estimation.

The general trend is clear, however, and prolonged partition of the more mobile fractions permitted fairly complete characterization of the tetra- to hexa-saccharides. Results of analyses of apparently individual spots resulting from a 10 days' partition of ethanol eluates collected between concentrations of 15 and 30% are given in Table IV. The values for glucose and fructose shown here were obtained by Somogyi titrations; when analyses of the individual spots from a duplicate chromatogram were made by means of the anthrone reagent, similar results were obtained. No successful complete separation of oligosaccharides higher than glucopentafructose has as yet been obtained.

Once the presence of this series of fructose-containing oligosaccharides had been established, it became of interest to determine the

actual amounts of the individual components in relation to the other sugars and oligosaccharides of the grain. Enzyme-inactivated barley was therefore exhaustively extracted with water, the solution was concentrated, gums and proteins were precipitated in the usual way, and the reducing values (glucose equivalents) of aliquot samples of the concentrate were determined by the Somogyi method. Mono-, di- and trisaccharides were estimated as relative glucose equivalents from chromatograms which had been partitioned for 3 days; gross values for fructosans were also determined on these 3-day chromatograms. For estimation of the individual fructosans, a second series of chromatograms from the same extract was partitioned for 10 days and estimations were then made of raffinose, glucodifructose, and six apparently separate fructosan bands, of lesser mobility than raffinose. Examination of the hydrolysis products of these fructosan bands suggested that they represented glucotrifructose, glucotetrafructose, glucopentafructose and three mixed bands giving glucose: fructose values respectively of 1:6·4, 1:7·2 and 1:7·5. The material on the starting line was contaminated by pentosan; after invertase hydrolysis the ratio glucose: fructose was 1:9·3 and traces of apparently pentosan material were left on the starting line.

After such prolonged partition, mono- and di-saccharides had travelled beyond the limits of the paper strip, but, by comparison with the amount of raffinose known to be present from results of the first estimation, approximate assessment of the individual fructosans was possible. Results of this determination are given in Table V. It must be emphasized that these results are only approximate; not only is aqueous extraction inadequate for complete removal of sugars, but the great dilution required for sugars such as sucrose, before colorimetric estimation can be applied, means that slight volumetric errors will lead to considerable errors in the final figures. These figures do, however, give a general picture of the distribution of the different types of fructose-containing oligosaccharides in the barley grain.

Discussion

It has been established beyond reasonable doubt that ungerminated barley contains, in addition to the simple sugars and oligosaccharides and the high-molecular fructosan, a series of compounds of intermediate molecular complexity, ranging from glucodifructose up to compounds of the order of molecular magnitude of 10 hexose units. Although the term "low-molecular fructosan" has conveniently been used to describe these oligosaccharides, it is something of a misnomer, for all appear to contain one glucose residue per molecule; however, since the more correct designation of monoglucopolyfructosan is rather cumbersome, no change is

TABLE V

Concentration of Sugars and Fructosan Fractions in Carlsberg Barley

Sugar or fructosan fraction	Glucose equivalents: mg. per 100 g. Analysis A	Analysis B
Glucose	51	—
Fructose	72	—
Maltose	143	—
Sucrose	828	—
Raffinose	316	316*
Glucodifructose (GF_2) ..	152	144
Fructosan fraction 1 (GF_3) ..	193	16
,, ,, 2 (GF_4) ..		27
,, ,, 3 (GF_5) ..		52
,, ,, 4 ($GF_{6\cdot7}$) ..		29
,, ,, 5 ($GF_{7\cdot8}$)		31
,, ,, 6 ($GF_{7\cdot9}$)		66
Material on starting line† ..	166	180

* Calculated to raffinose value obtained in A see text.

† Faint pink colour with aniline oxalate; probabl pentosan contamination.

proposed in nomenclature. Their susceptibility to invertase indicates that thes fructosans are formed from β-fructofuranc sidic residues, and it is probable that the comprise an homologous series, based o sucrose. Barley, then, closely resemble other fructosan-containing plants in possess ing intermediate compounds which form stepwise progression from sucrose to th highest polyfructosan, which is itself te minated by sucrose. Though the constituer sugars have been identified and their pr portions estimated for a number of th different individuals of the series, the fu chemical constitution of these fructo polymers has not yet been established. fructosan from barley leaves, isolated b

Archbold & Barter[3] and examined chemically by Haworth, Hirst & Lyne[12] has been shown to contain about 10–12 β-2·6-linked fructofuranoside residues. It cannot lightly be assumed that the fructosans of the ripe grain are identical with those from the leaves of the same plant, but the degree of continuity apparent in the accumulation of fructosans in the flag leaf and its sheath, the upper internodes of the stem and the immature grains,[1] suggests that the same series of compounds may be present in all these different organs of the barley plant. To settle this point conclusively, however, a full chemical examination of purified samples of the grain fructosan would be necessary.

The exact metabolic significance of the fructosans is still imperfectly understood. Accumulation of fructosan in barley stems appears to occur when sugars translocated from the leaf to the internode are in excess of immediate requirements[1]; the fructosans of the immature ear may represent a similar type of temporary storage, which is gradually utilized to provide raw materials for respiration in the ripening ear. The central position of sucrose in barley germination has already been discussed,[19] and the fructosans would appear to offer a readily-available supply of sucrose. Edelman & Bacon have shown that extracts of artichoke tubers can effect not only simple hydrolysis of fructosans,[10] but also a process of transfructosidation,[11] thus:

$$(\text{fructose})_n + \text{sucrose} \rightarrow (\text{fructose})_{n-1} + \text{glucodifructose}.$$

Whether or not a similar mechanism for interconversion of fructosans is present in barley remains to be seen, but the existence of some such metabolic pathway seems highly probable; fuller understanding of the formation and utilization of the barley fructosans will best be achieved through studies of the relevant enzyme systems.

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RAFFINOSE METABOLISM IN GERMINATING BARLEY

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(With 1 figure in the text)

The trisaccharide raffinose is widely distributed throughout the Spermaphyta (French, 1954), not infrequently accounting for a substantial proportion of the free sugars of seeds, rhizomes or roots. Structurally, raffinose may be represented as α-D-galactopyranose (1→6) α-D-glucopyranose (1→2) β-D-fructofuranose; oligosaccharides related to raffinose include the tetrasaccharide stachyose which contains a second galactose unit joined by an α(1→6) linkage to the terminal galactose residue of raffinose, and the pentasaccharide verbascose. These oligosaccharides resemble one another in being terminated by sucrose and in containing a number of α(1→6)-linked galactose units.

In barley the only one of this group of oligosaccharides which has been identified with certainty is raffinose itself. First recorded from ungerminated barley by O'Sullivan (1886), raffinose in cereals has attracted considerable interest and numerous analyses are now available for the total quantities of the sugar present in the entire grain (see, e.g. MacLeod, 1952) and in the isolated embryos (James, 1940). The average amount of raffinose in intact barley corns is of the order of 0.5 per cent of the dry weight, though figures ranging from 0.14–0.83 per cent have been recorded. Raffinose normally accounts for about one-quarter of the total free sugars and low-molecular fructosans of the ungerminated grain. The distribution of the sugar within the corn is not, however, uniform. Thus, Colin and Belval (1933) could not detect raffinose from barley flour, though the embryo contained 4 per cent of the sugar, and MacLeod (1952), in an examination of differentially-milled fractions of barley, reported 3.6 per cent of raffinose from the fraction containing the germ, compared with only 0.008 per cent from the pearled grain, which is almost exclusively endosperm. In barley, therefore, raffinose appears to be associated predominantly with the living meristematic tissues of the embryo.

When barley germinates, raffinose disappears from the developing seedling. Thus Kluyver (1914) failed to detect raffinose from partly grown barley malt, though this sugar accounted for 0.45 per cent of the unmalted grain, and James (1940), in a detailed analysis of sugars from excised embryos and seedlings of Plumage-Archer barley, found that raffinose had completely disappeared from the young plant after 24-hour growth at 21° C. On the other hand, MacLeod, Travis and Wreay (1953), using chromatographic methods of separation to follow changes in the sugar content of Ymer barley during commercial malting, were able to demonstrate the continued presence of raffinose for approximately 4 days after the wetting of the grain. A similar pattern of slow utilization of raffinose is also apparent in results quoted by Harris and MacWilliam (1954) for a commercially-malted Spratt-Archer barley. The delayed mobilization of raffinose during malting compared with the very rapid disappearance of the sugar from laboratory-grown material was initially attributed to the lower temperatures prevalent during the

malting process, but more recently results of laboratory germination tests designed to study commercial malting at higher temperatures have shown that even at 21° C. raffinose can persist in germinating corns for 2-3 days. Clearly, then, temperature is not necessarily the decisive factor determining the different rates of utilization of raffinose.

In malting, barley is first steeped for approximately 72 hours, normally with some degree of aeration and with one or two changes of steeping liquor, before being cast on to the malting floor, where controlled growth of the seedling takes place; in the determinations of sugars from barley during malting the slow rate of raffinose utilization coincided with the period of steeping. On the other hand, in the work reported by James (1940) the corns were grown on moist sand with their embryos freely exposed to air from the inception of the experiment; under these conditions raffinose utilization was apparently immediate and rapid.

Steeping may influence barley not only by making available to the embryo a greater supply of water than can be imbibed from a moist substrate, but also by imposing on the embryo at least partial anaerobiasis. It seemed of interest, therefore, to compare the rate of utilization of raffinose by barley grown aerobically at 21° C. with that of barley steeped at 21° C., and also to examine raffinose metabolism in grain in which aerobic respiration was impeded directly through the inhibition of a terminal oxidase.

During the first 24-hour growth, simultaneously with the disappearance of raffinose from the embryo, structural polysaccharides are synthesized (James, 1940). The easily-hydrolysed polysaccharides are likely to comprise hemicelluloses and pectic materials, including galactan, and remarkably little is known about the precise mode of synthesis of these materials. In addition to the principal investigation, therefore, attempts were made to fractionate the polysaccharides of the embryo in order to determine if there was any correlation apparent between loss of raffinose and synthesis of galactan.

Materials

The grain used throughout was a sample of 1954 Carlsberg barley grown in East Lothian. At receipt the moisture content was 12.4 per cent and the germinative capacity 98.5 per cent; after eighteen months' storage in a bin at room temperature, while the moisture content had risen by only 0.5 per cent, the germinative capacity had fallen to 91 per cent. Corns with obviously damaged embryos were excluded from the analyses but loss of viability cannot be detected after a few hours' exposure to conditions appropriate for germination, so approximately 9 per cent of dead corns may unwittingly have been included in the later analyses. As various samples from all series of tests were included in the later analyses, and as many of the determinations were replicates of earlier work, this diminished viability has extended over the whole range of results. While this must undoubtedly lead to loss of precision it has not caused bias in any particular direction.

Methods

Preliminary studies were carried out with embryos excised from intact corns. This excision involves preliminary removal of the husk and the pericarp-testa and is rather time-consuming, so a less laborious preparative method was investigated. Pollock, Essery and Kirsop (1955) have advocated a 3-hour steep in 50 per cent sulphuric acid for overcoming dormancy in barley, a treatment which removes husk and pericarp while

leaving the testa intact. The embryos can then be rapidly excised and, surprisingly enough, germinative capacity is unimpaired by this drastic procedure. Presumably osmosis occurs between the interior of the grain and the acid steep, so that results obtained with grain treated in this manner would not be strictly comparable with those from untreated material. All results reported here are from acid-treated grains.

Steeping technique

Approximately 110 corns were placed in tap water in a Hirsch tube which was subjected to three successive evacuations at the pump, each followed by re-admittance of air. Small bubbles formed at the apices of the corns during evacuation and escaped into the surrounding water. The corns were then immersed in 100 ml. of freshly boiled tap water which had been covered with a film of oil and cooled to 21° C., and were maintained in the dark at that temperature for the desired length of time. 100 embryos were excised at intervals over 24 hours and dropped into boiling 80 per cent ethanol, to inactivate enzymes and initiate sugar extraction. Three 30-min. extraction periods under reflux, followed by a final wash with 80 per cent ethanol, sufficed to extract all free sugars from the embryos and the combined ethanolic extracts were stored till required. The sugar-free embryos were dried and reserved.

Germination

For aerobic treatment it was considered desirable to use conditions which, while limiting the amount of liquid water available, would promote regular growth. Since the present study was restricted to the first 24-hour growth of the seedling, during which time no real irregularities of behaviour can be appreciated, various techniques were first investigated with corns grown for 72 hours, with a view to selecting a method which would give maximum regularity of growth. It is assumed that seedlings which are growing uniformly at 72 hours have all passed through similar stages during the first 24 hours after wetting the corns.

Growth on sand proved unsatisfactory, not only because sand particles adhered to root hairs and proved an embarrassment in subsequent estimations of seedling dry weight, but also because pockets of excess moisture tended to collect locally and rootlet growth varied greatly from corn to corn. Eventually, a simple technique, modified from a method described by Massart (1955) for embryo culture, was found to give consistent and regular seedling growth from the treated corns. In this method a 15×15 cm. glass plate with a central hole was placed over a petri dish and covered by a filter paper from which a radial tongue had been cut. The tongue passed through the hole into tap water in the petri dish and a flow of water was created over the filter paper in a manner reminiscent of the development of a circular paper chromatogram. Corns were placed on the filter paper with the embryos uppermost and a saturated atmosphere was maintained by covering the corns with a second petri dish. Excised embryos were killed and extracted by the same methods as were used for steeped grain.

Oxidase inhibition

Cytochrome oxidase acts as the principal terminal oxidase of young barley seedlings (James, 1953), and this metallo-enzyme can be readily inhibited by cyanide. For the treatment with cyanide, 100 corns were maintained aerobically on irrigated filter paper for 2 hours and then injected with 10^{-3}M KCN at the pump and left in the cyanide

solution for 2 hours. For the next 20 hours the corns were placed on filter paper irrigated with 10^{-3}M cyanide from the lower petri dish in the usual aerobic germination assembly. Excision and sugar extractions were carried out in the normal manner, and, as a control, comparison was made with grains treated with tap water in place of KCN.

Sugar analyses

For all determinations of sugars, extracts were freed from ethanol by distillation, filtered and made to 25 ml. Aliquots were treated with BDH invertase concentrate for 2 hours at pH 4.6, preliminary chromatographic studies having shown that this treatment sufficed to hydrolyse sucrose and to convert raffinose completely to melibiose and fructose. Hydrolysis products were estimated by the Somogyi (1945) technique. The remainder of the extract was concentrated and separated chromatographically on paper using as solvent the upper layer of a mixture of butanol (40), acetic acid (10) and water (50). Sugars were eluted from the appropriate regions of the chromatograms and hydrolysed by invertase, and hydrolysis products were again estimated by the Somogyi microcopper method. The relative quantities of the hydrolysis products of the individual sugars could then be determined, and by reference to a previously-prepared standardization curve the absolute amount of raffinose could be ascertained. The use of invertase in place of the more conventional acid hydrolysis avoided destruction of fructose in the prolonged acid treatment needed for complete hydrolysis of raffinose, and, because of the smaller number of transfers of solutions required, lessened the risk of mechanical losses of sugars. The degree of accuracy possible with the method can be gauged from Table 1, where results of analyses by this method of mixtures of known concentrations of raffinose and sucrose are shown.

Estimation of polysaccharides

Attempts were made, unsuccessfully, to extract hemicelluloses from the residual sugar-free seedlings by NaOH, but recoveries were small and very variable. However, a crude estimate of easily-hydrolysed polysaccharide was achieved by grinding the dried residual seedlings with celite, hydrolysing for $4\frac{1}{2}$ hours with N H_2SO_4 and determining hydrolysis products, after neutralization, by the usual microcopper method. Chromatographic separation of this hydrolysate was carried out in butanol (90), ethanol (10), water (100).

In order to achieve a fuller carbohydrate analysis of the embryos from ungerminated grain, one determination of the more resistant polysaccharide was carried out. The residual material from the mild acid hydrolysis was dried and subjected to the action of 72 per cent H_2SO_4 for 4 hours, and after dilution of the acid to approximately 2N, was boiled under reflux for $4\frac{1}{2}$ hours. The total sugar concentration in the hydrolysate was then determined.

Infiltration of embryos with raffinose

In a preliminary study of the effects of high concentrations of raffinose on the metabolism of the embryo, grains were grown aerobically for 15 hours, and their excised embryos were then injected with a saturated solution of raffinose. The embryos were then supplied with raffinose from the lower petri dish in the usual aerobic germination assembly. Sugar analyses were carried out at 2-hour intervals after the initial introduction of raffinose.

RESULTS

Sugar utilization

The only sugars detectable on chromatograms from embryos excised from grain which had been grown for 2 hours (the earliest time at which excision was possible) were sucrose and raffinose. After 24 hours' aerobic growth, small quantities of fructosans were apparent and raffinose had completely disappeared, but no hexose or maltose could be detected in the seedlings during this first day's growth. As can be seen from Fig. 1A,

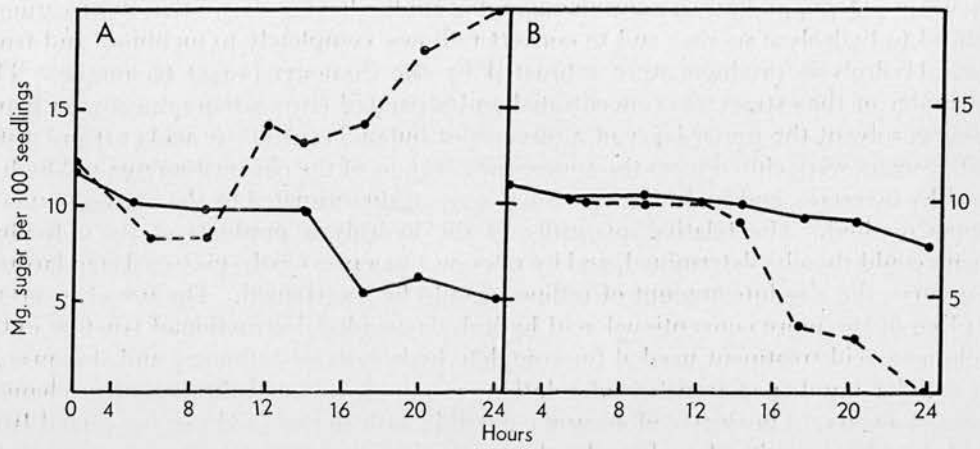

Fig. 1. Sugar utilization by barley seedlings. A, sucrose. B, raffinose. Broken line, germination in air. Unbroken line, grains immersed in water.

sucrose concentration fell during the first 9 hours and thereafter rose steadily. This rise was rather unexpected as it had been assumed that translocation from the endosperm would not occur during the first 24 hours' growth. However, analyses of the residual endosperms revealed that substantial reserves of sucrose (*ca.* 14 mg. per 100 endosperms)

Table 1. *Analysis of mixtures of known amounts of raffinose and sucrose*

Sugar taken in 25 ml. solution (mg.)		Sugar found (mg.)
Raffinose	7.2	6.7
Sucrose	18.7	19.0
Raffinose	9.5	8.6
Sucrose	11.0	11.6
Raffinose	2.5	1.8
Sucrose	22.0	23.0

were present in the ungerminated grain; this endospermic sucrose diminished gradually — presumably as a result of translocation — from the 6th hour after the grain came into contact with water. No raffinose was found in the endosperm, and the raffinose in the embryo fell from 10.6 mg. to zero in approximately 24 hours. It is notable, however, that the mobilization of raffinose did not begin until some 14 hours after the grain had been wetted, during which period substantial inroads had been made on the endogenous

sucrose of the embryo, and further supplies had appeared, presumably from the endosperm.

With the steeped grain, the disappearance of sucrose from the endosperm occurred at the same rate as in the aerobically grown material, but the actual consumption of sucrose by the embryo was much greater, so that the curve for sucrose in the anaerobic embryo (Fig. 1A) merely flattens out after 16 hours. Raffinose (Fig. 1B) was obviously still present in the embryos after 24-hour steeping.

At the end of the 24-hour steeping period the corns were capable of vigorous growth when transferred to aerobic conditions, and after 12 hours in the normal aerobic germination chamber raffinose had disappeared from the embryos. With more prolonged steeping (40 hours) 5 mg. raffinose still persisted per 100 embryos, but with material steeped for such a long time subsequent growth in air was slow and only about one-half of the seedlings developed normal rootlets. No further investigation was made of prolonged steeping treatments.

Losses of sugars from the grain to the steep liquor were small — of the order of 3 mg. sugar per 100 corns during 24 hours' immersion, and qualitative chromatographic examination of concentrates showed the presence of the whole spectrum of sugars normally found in ungerminated barley — fructosans, raffinose, maltose, sucrose, glucose and fructose — so that dissolution of raffinose from the embryo must have been insignificant. With the grains grown aerobically elution of sugars to the filter paper was negligible.

Sugars from cyanide-treated seedlings

Embryos excised from the control contained sucrose as the only free sugar after 24 hours, whereas, in the embryos which had been exposed to cyanide, raffinose was clearly present. Replicate determinations from cyanide-treated material showed greater variations in the absolute quantities of sugars present in the embryos; typical results are given in Table 2. When corns which had been treated with cyanide were washed, transferred to filter paper and supplied with water, the coleoptile emerged normally, though rootlet development was poor.

Table 2. *Sugars from cyanide-treated embryos*

	Raffinose	Sucrose
	mg. per 100 embryos	
(*a*) Cyanide-treated	4.4 5.2 3.6	4.3 5.6 5.9
(*b*) Controls	0 0	13.7 12.8

Polysaccharide synthesis

All chromatograms of hydrolysates revealed the presence of galactose, glucose, arabinose and xylose, in quantities sufficient for elution and estimation; traces of galacturonic acid were also apparent, but the amounts present were too small for determination. No increase in polysaccharides was detected in the steeped material over the 24 hours under consideration, but with the aerobically grown corns, the amount of synthesis

by the embryo was appreciable. Changes in the amounts of the sugar units present are given in Table 3; the figures quoted there are glucose equivalents and each represents the mean of three determinations.

Table 3. *Polysaccharide synthesis. Sugar units after hydrolysis* (*mg. per* 100 *seedlings*)

Germination (Hours)	Easily-hydrolysed polysaccharide				Resistant polysaccharide
	Galactose	Glucose	Arabinose	Xylose	
2	1.2	2.7	4.8	2.4	3.3
17	1.3	2.1	4.5	2.6	—
24	3.0	4.8	4.6	2.7	—

Sugar utilization in isolated embryos

In view of the complications introduced by the unexpectedly massive translocation of sucrose from the endosperm at an early stage in seedling development, an assessment was made of the total utilization of sugars by isolated embryos in 24 hours, together with an analysis of the easily hydrolysable polysaccharide synthesized in that period. Growth was on moist filter paper, in the usual manner.

Table 4. *Carbohydrate changes in isolated embryos* (*mg. per* 100 *seedlings*)

Germination (Hours)	Glucose	Sucrose	Raffinose	Easily-hydrolysed polysaccharide
2	0	13.0	9.5	11.1
24	0.4	1.0	1.5	13.1

Results of these determinations are given in Table 4 and for comparative purposes they are again quoted as glucose equivalents. It may be noted that, with isolated embryos, raffinose was still definitely present, though in greatly diminished amount, after 24 hours' growth.

Raffinose infiltration

After 2 hours, embryos which had been supplied with saturated solutions of raffinose were found to contain an additional oligosaccharide of lesser chromatographic mobility than raffinose, together with traces of galactose. With more prolonged treatment, the amounts of these sugars increased but no further oligosaccharides were observed during the 8 hours over which observations were made, though, owing to the high concentrations of raffinose employed, trace quantities of other sugars might have evaded detection on paper chromatograms. The synthesized oligosaccharide was non-reducing and gave a ketose reaction with α-naphthol in phosphoric acid. A small amount was eluted from 3 MM paper and hydrolysed with N HCl in a sealed tube for 4 hours at 100° C., and, after taking to dryness and separation in butanol-ethanol-water, the hydrolysate was found to contain galactose, glucose and fructose. From its position on the chromatogram this oligosaccharide appeared to be a tetrasaccharide and, although the amounts recovered were not sufficient to allow quantitative estimation of the sugar units present, visual examination of the sprayed chromatograms of hydrolysates indicated that galactose was the predominant hexose. The oligosaccharide therefore resembled stachyose.

Discussion

The embryos of the sample of Carlsberg barley investigated contained, on a dry-weight basis, 9 per cent of raffinose, 11 per cent of sucrose, 9 per cent of easily hydrolysed polysaccharides and 3 per cent of predominantly cellulosic material. Apart from the fatty material undoubtedly present, the sugars and possibly also part of the easily hydrolysed polysaccharide fraction constitute the primary substrates for embryo respiration and for synthesis of the cell-walls of the growing seedling; it is worthy of note that this labile material accounts for approximately one-fifth of the dry weight of the embryo and for over 60 per cent of the total carbohydrate therein.

The basic pattern of sugar utilization in the Carlsberg sample was essentially similar to that reported by James (1940) for Plumage-Archer barley. Thus, 100 isolated embryos grown aerobically for 24 hours mobilized 12 mg. of sucrose and 8 mg. of raffinose, with concomitant synthesis of 2 mg. of easily-hydrolysed polysaccharide; the Plumage-Archer sample grown under rather similar conditions metabolized 15.2 mg. of sucrose and 4.8 mg. of raffinose while 3.6 mg. of 'hemicelluloses' were synthesized. The general similarity of behaviour is not apparent where intact corns are involved since in the Carlsberg barley, translocation of sucrose from endosperm to embryo six hours after the inception of the experiment obscured the fall in sucrose in the developing seedling.

Once again, with aerobically-grown intact corns, raffinose could not be detected in the embryo after one day's growth. There is, however (Fig. 1B), an apparent delay in raffinose utilization for approximately 14 hours after the grain is transferred to moist filter paper. It may be significant that determinations of respiratory quotients (James and James, 1940) indicate that oxygen uptake by the embryo is impeded for approximately this length of time; the temporary oxygen block is believed to be due in part to the formation of a film of water round the grain.

No delay occurred in the utilization of sucrose under aerobic conditions, and with steeped grain (Fig. 1B) there is clear evidence of the failure of the embryo to metabolize raffinose during a period when sucrose consumption was rapid. With steeped material, indeed, no clear-cut utilization of raffinose occurred during the 24 hours of the experiment, though the slight decline in concentration may have resulted from a genuine metabolism of raffinose by the embryos. It will be remembered that, despite the supposedly semi-permeable nature of the testa, small quantities of sugars were leached into the steeping medium and, with the high initial concentration of raffinose in the embryo, it is not impossible that some raffinose may have diffused into the empty cells of the proximal part of the endosperm. Admittedly, examination of endosperm extracts failed to reveal any raffinose, but the presence of considerable quantities of fructosans, together with the low concentrations of raffinose involved, made detection difficult.

Whether the minor loss of raffinose from embryos of steeped grain was merely physical or truly biological, it is abundantly clear that, while sucrose is metabolized efficiently both by steeped grain and by grain grown with maximum aeration, raffinose is metabolized only to a limited extent in steeped grain. Moreover, from the results obtained with cyanide-treated grain (Table 2), where again raffinose utilization in 24 hours was incomplete, in contrast to the enhanced consumption of sucrose, it would appear that inhibition of the cytochrome system also at least partially inhibits utilization of raffinose.

In the absence of a detailed analysis of sugar fluctuations during the first day of growth, it was reasonable to assume that raffinose and sucrose were equally effective substrates for respiration; indeed, since the disappearance of raffinose from the embryos

of intact corns was complete within 24 hours it could plausibly be suggested that raffinose was the preferred initial substrate. However, in view of the results presented above, some re-assessment of the situation is desirable. It would now appear that, in barley embryos, raffinose cannot be metabolized unless normal respiration is in progress; sucrose, on the other hand, is consumed, rapidly in anaerobic conditions such as occur when seeds are immersed in water or when terminal oxidases are inhibited, and in a more leisurely fashion during normal aerobic growth. The preliminary stages of carbohydrate breakdown occur readily in the absence of oxygen, and it would seem that, with steeped barley, the sucrose in the embryo provides an adequate substrate for glycolysis whereas the raffinose cannot be immediately incorporated into the glycolytic sequence. This is rather unexpected, since raffinose actually has sucrose forming part of its molecule; the failure to metabolize raffinose can only be due to the influence of the additional terminal galactose residue.

Neither galactose nor melibiose was detected at any time in untreated barley embryos, even after prolonged steeping, and it must therefore be inferred that α -galactosidase and β -fructofuranosidase are absent or that, if present, these enzymes are in some way segregated from the potential substrate, raffinose. However, in addition to possible galactosidases, other enzyme systems have recently been described which may be of considerable importance in relation to the metabolism of galactose and galactosides. One such system involves the nucleotide, uridine diphosphate (UDP), as a carrier of high-energy phosphate groups. Cardini, Leloir and Chiriboga (1955) have shown that preparations from wheat germ and from barley seedlings can catalyse the reaction

$$\text{UDP-glucose} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{UDP}.$$

With a ΔF of approximately -1000 the equilibrium of this reaction is in favour of sucrose synthesis. As well as promoting the above reaction, UDP-glucose acts as a co-enzyme to 'galactowaldenase' in the inter-conversion of glucose-1-phosphate and galactose-1-phosphate with presumed intermediate formation of UDP-galactose; the supply of UDP-glucose is maintained at the expense of UTP and glucose-1-phosphate (Leloir, 1953). If some system of this nature is concerned in raffinose metabolism — and it is one of the very few enzyme systems which have been shown to involve galactose — then the need for oxygen to maintain the supply of high-energy phosphate bonds is more readily understood. In passing, it is of interest to note that the list of seeds in which UDP-glucose has been identified (Cardini *et al.*, 1955) largely coincides with the list of seeds known to contain members of the series raffinose, stachyose, verbascose (French, 1954).

Again, from the results obtained with isolated embryos, it would appear that raffinose is fully metabolized only in conditions in which synthesis is taking place. It will be recalled that Brown and Morris (1890) found that while isolated embryos supplied with sucrose were capable of adequate growth, substitution of raffinose for sucrose allowed only insignificant increase in the dry weight of seedlings fed with the sugar. In the present study, and also in the much more extensive investigation of James (1940), raffinose did not completely disappear from embryos which had been separated from their endosperms. Indeed, though raffinose concentration fell to approximately 1 mg. per 100 embryos in 24 hours, it was still present at the conclusion of James's experiments after 6 days. Synthesis of polysaccharide had ceased after 24 hours, sucrose had vanished from the embryo, hemicelluloses were undergoing degradation, CO_2 emission was declining — but trace quantities of raffinose remained. This failure to metabolize the

last traces of raffinose in conditions of virtual starvation is at first sight rather puzzling. If, however, as was tentatively suggested above, mobilization of raffinose involves prior synthesis of high-energy phosphate bonds, then the persistence of raffinose when other suitable respiratory substrates are greatly depleted is more readily understood.

The nature of the polysaccharides synthesized during the period of the experiment gave no clue to the possible origins of the sugar units. The increase in hemicellulosic material in aerobically-grown seedlings was mainly due to synthesis of galactan and glucosan (Table 3) and though the increase in galactose residues was considerable it was very much less than would be expected if raffinose (or all the galactose from it) was directly incorporated into the polysaccharide. Again, though careful search was made for galactose-containing oligosaccharides of greater molecular complexity than raffinose, none could be found from normal seedlings even during the period of most rapid utilization of raffinose. Certainly, excised embryos injected with a saturated solution of raffinose were able to form a tetrasaccharide with the chromatographic characteristics and the hexose constitution of stachyose, but no more highly elaborated material than this could be found, though free galactose was released. The fact that a tetrasaccharide was synthesized in presence of abnormally high concentrations of raffinose does not provide any clear indication as to the means by which the bulk of the raffinose in normal embryos is metabolized; the sudden drastic change in the equilibrium of the sugars concerned may have brought into prominence a metabolic route which is normally only of minor importance.

While no information is available about the structure of barley galactan, Hirst, Jones and Walder (1947) have investigated a pure galactan isolated from seeds of *Lupinus albus* and have shown that it consists of β (1→4) linked galactose units. A galactan of this nature could not be formed by direct transglycosylation from known galactose-containing oligosaccharides, as all the simple galactosides from plants appear to contain α (1→6) linked galactose residues (French, 1954). Though raffinose (and its higher homologues) and galactan frequently co-exist in seeds, there is little evidence for synthesis of one class of compound from the other; it is more probable that these two types of material containing galactose residues are unrelated, apart from their derivation from a common metabolic pool.

The precise nature of the mechanism controlling raffinose utilization remains obscure but it would appear that, in barley seedlings, raffinose is metabolized only when some other suitable respiratory substrate such as sucrose is available and when normal aerobic respiration is in progress.

Summary

(1) Raffinose was absent from the endosperms of the sample of Carlsberg barley investigated but accounted for 9 per cent of the dry weight of the embryos.

(2) When corns germinated in air on moist filter paper there was a preliminary lag of 14 hours during which raffinose concentration remained unaltered. Subsequent utilization of raffinose was rapid and no raffinose could be detected in seedlings excised from intact grains after 24 hours' growth. With isolated embryos, however, raffinose utilization was incomplete in 24 hours.

(3) When barley was immersed in water 70 per cent of the raffinose initially present remained unattacked after 24 hours and, in presence of cyanide, raffinose utilization was again incomplete.

(4) Although synthesis of a tetrasaccharide could be induced by injection of concentrated solutions of raffinose into barley embryos, no evidence was found for direct incorporation of raffinose into more complex carbohydrates during normal seedling growth.

(5) It is concluded that the raffinose of barley embryos is metabolized only when some other suitable respiratory substrate is present. Aerobic respiration is essential for consumption of raffinose.

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COMPARATIVE STUDIES OF EMBRYO AND ENDOSPERM

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COMMUNICATIONS

COMPARATIVE STUDIES OF EMBRYO AND ENDOSPERM

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Results of chemical analyses of wheat germ and of barley endosperm indicate that, while the germ preparation can yield typical pectic materials, including a pure araban, such materials are absent from the central endosperm. The behaviour of sections of seeds of various grasses in a number of different solvents indicates that in all samples examined of *Bromus sterilis*, and in some samples of barley, intercellular cementing material is either lacking or else very readily soluble in water, whereas in all samples examined of *Bromus mollis* and in other samples of barley, proteinaceous material is present between the individual cells of the endosperm.

Introduction

Polysaccharide analyses.—Systematic studies carried out during the past ten years by Preece and his collaborators have successfully solved a number of problems relating to the non-starchy polysaccharides of barley and other cereal grains. Water-soluble[16] and NaOH-soluble[12] hexosans and pentosans have been examined, a pure β-glucan has been separated from barley[16] and from oats,[11] and quantitative determinations of water-soluble β-glucan have shown that this material is virtually eliminated during malting.[10] Furthermore, enzyme systems capable of catalysing degradation of β-glucan have been intensively studied[14] and preliminary results of investigations relating to the enzymic breakdown of pentosans have been published.[13,15] Much of this work has been concerned with the water-soluble material, but a comparison of these so-called cereal gums with the NaOH-soluble hemicelluloses[12] suggests that the water-soluble fraction can best be classed along with the typically water-insoluble hemicelluloses, which may, indeed, partially serve as gum precursors during the early stages of malting.

Much, then, has been accomplished, and though the early hopes of utilizing knowledge of glucan and glucanase concentration to predict behaviour on the malting floor have not been fully realized,[10] the theoretical knowledge amassed has laid a useful foundation for a more complete understanding of carbohydrate modification during malting. It is salutary, however, from time to time to look back on accomplished work, not merely in admiration but with a view to discovering omissions and anomalies, and to consider results which have been obtained with a rather narrow range of plant materials against the whole corpus of knowledge of plant biochemistry.

Considered as a small part of an infinitely larger whole, the cereal polysaccharides present several interesting features. β-glucan has been detected in various wild grasses more or less closely related to barley,[7] and in oats,[11] but the only other known source of polysaccharide resembling β-glucan is not a flowering plant but a lichen, *Cetraria islandica*.[18] The lack of a record of any given material in a plant may indicate either that the material is indeed absent or that it has never been adequately sought, and obviously it cannot be definitively stated that β-glucan, or material resembling it, is present only in grasses and in a lichen. However, investigations carried out in this Department and covering a range of seeds from plants belonging to six different widely-separated families have failed to reveal any trace of β-glucan. The β-glucan of certain cereal seeds is therefore, for the present, something of a curiosity.

The pentosan fraction of grass-seed hemicelluloses is also rather unusual. Water-soluble hemicelluloses precipitated by ammonium sulphate from extracts of cereal seeds are virtually free from uronide, and even

in the NaOH-soluble material prepared from pearl barley and from wheat flour no uronic acids could be detected.[12] This absence of uronide from the hemicelluloses is certainly unusual; Whistler & Smart[18] state that "while information regarding the constituents of hemicelluloses is fragmentary . . . uronic acids have been detected in practically all."

It would appear, then, that certain non-starchy polysaccharides of cereal grains are, if not unique, at least not directly comparable with hemicelluloses from other sources. It is generally accepted that the hemicelluloses of woody tissues are located in the cell walls, in more or less intimate contact with cellulose and lignin, and by analogy it is tacitly assumed that the hemicelluloses of grass seeds—pentosan and glucan—are also cell-wall constituents. It may be wondered whether there is any real justification for this assumption, especially in the case of β-glucan, which does not figure prominently in any other hemicellulose preparation.

The virtual absence of uronic acids from certain preparations from cereal endosperms is also of interest in that it suggests that these products are free from pectic materials. Pectic acid is frequently present as a calcium or magnesium salt and the sodium hydroxide treatment used to solubilize hemicelluloses should certainly have revealed at least a proportion of any calcium pectate present. No hydrolysis products ascribable exclusively to pectin were detected in endospermic hemicelluloses,[12] and since certain preparations made from whole grains undoubtedly contained galacturonic acid, it would appear that any pectin present in the grain is extra-endospermic.

Associated with derivatives of polygalacturonic acid in crude samples of pectin there are almost invariably found substantial quantities of galactan and araban; indeed the recognition of arabinose and galactose in hydrolysates is often indicative of the presence of pectin. Galactan has not been reported from purely endospermic products, and the proportions of arabinose present there are probably sufficient only to provide for the arabofuranose known to constitute side chains of the endospermic hemicellulose. In certain preparations of low molecular weight derived from intact grains, however, galactan is unequivocally present[11] and araban is considerably in excess of the usual quantities found in xyloarabans. There is, therefore, an indication of the presence both of polygalacturonic acid and of the usual pectic concomitants in the crude polysaccharide fraction of low molecular weight prepared from intact cereal grains, although the total amount of pectin present would appear to be very restricted.

Anatomically, pectin is generally considered to constitute the so-called middle lamella of plant tissues; when a meristematic cell divides, a "cell plate" forms between the daughter nuclei, pectic materials are deposited on this cell plate and the middle lamella so formed remains in the tissue till senescence, gumming the cells together. Middle-lamella pectin is said to be particularly abundant in young tissues, and it would be reasonable to expect a cereal seed, with a completely meristematic embryo and an unlignified endosperm, to prove a rich source of pectin. The embryo of cereals, however, accounts for a very small proportion of the total weight of the seed and, as suggested above, it would seem to be the quantitatively more important endosperm which is so strangely devoid of the sugar units normally derived from pectin. It may be helpful, therefore, to consider something of the early history of grass endosperms.

Endosperm formation.—Endosperm has been described as a peculiar tissue with three parents and no future. It originates from the characteristic double fertilization in which two of the haploid female nuclei are conjoined with the second male gamete, thus giving a triploid nucleus, the endosperm mother nucleus. This nucleus divides rapidly so that, in wheat for example, 8–16 endosperm nuclei may be present at the time of the first division of the zygote.[1] While the nuclei formed from the zygote behave in the usual fashion characteristic of dividing meristematic cells, with formation of a cell-plate and middle lamella, the nuclei formed from successive endosperm nuclear divisions act quite anomalously. Initially no cell walls at all are present. The nuclear divisions tend to occur synchronously and the free nuclei line the cavity of the embryo sac, each nucleus accompanied by a circumambient ball of cytoplasm—but no cell walls are apparent. This free-nuclear phase ends abruptly about $3\frac{1}{2}$ days after pollination, and by 4 days the whole endosperm is

cellular. The precise mechanism involved in laying down these first cell walls does not seem to have been described. As the endosperm matures, the innermost cells do not normally divide again but they enlarge considerably and become filled with starch granules. About three weeks after pollination the outer layers become quiescent and are transformed to aleurone, and cell division continues sporadically in the sub-aleurone layer. Bi-nucleate cells are not uncommon in the endosperm of barley,[2] so that, even in the predominantly cellular phase of endosperm development, cell-wall formation may not follow the regular course typical of, for example, a growing rootlet.

Pectin.—Reverting now to the distribution of pectin in cereal seeds, it can be seen that the cytological abnormalities apparent in at least the early development of the endosperm are sufficiently great for the absence of normal concomitants of cell-wall formation, such as pectin, to appear less surprising. The evidence for absence of pectin, however, is mainly circumstantial, taken from results of investigations dealing not with pectin as such, but with other possibly related carbohydrates, and, though circumstantial evidence is useful where no direct information is forthcoming, direct estimations of pectin, specifically sought for, would be more convincing. On consulting *Modern Cereal Chemistry*,[5] however, it is found that neither pectin nor polygalacturonic acid features in the Index—an omission which suggests that pectic materials have never raised the faintest stir in the world of cereal technology. Again, if the Index of this *Journal* for the past ten years can be taken as a reliable guide, it seems that, apart from academic work relating to the constitution of pectin and a few papers on the pectin content of hops, pectic substances have not been considered worthy of the attention of the maltster or the brewer. Nanji & Norman[9] certainly reported 1% of "pectin" from barley, but Just[4] later suggested that this "pectin" must have been very impure and reached the conclusion that, although barley husk yielded 0·04% of material containing galacturonic acid, no significant amount of pectin was present in whole barley.

If then, as far as can be judged, pectin and its concomitants are of no importance to the cereal technologist or to the maltster, why bother about the distribution of an infinitesimally small amount of this ill-characterized material, generally admitted to be difficult to prepare, impossible to purify and of incomprehensible function? The justification of a further investigation is that, if pectin is indeed absent from the endosperm, the questions arise: what forms the intercellular cement of this tissue, and what mechanism is involved in the dissolution of the intercellular material—if any—in cereal endosperms during germination?

With a view to answering these questions, two lines of approach were followed: (*a*) a chemical investigation of specific, botanically homogeneous tissues representative of different regions of the grain; and (*b*) a histological survey of endosperm cells with particular reference to the cell walls and the possible middle lamella.

Experimental

Chemical studies.—The cereal preparations chosen for detailed analysis were pearl barley, representing purely endospermic material, and wheat germ. These materials were selected because similar products had earlier been examined by Preece & Hobkirk for hemicellulose content[12] and also because they appeared to offer the most homogeneous sources of the tissues under consideration. Wheat flour, also a purely endospermic material, was used in some preliminary work, but the technical difficulties involved in preparing extracts were so great that further work with this material was abandoned.

Water-soluble and NaOH-soluble hemicelluloses were first prepared for comparison with polysaccharides previously extracted from samples of similar material. Extraction was carried out in the manner described by Preece & Mackenzie[16] and the hemicelluloses were precipitated by Fehling's solution and acetone, and examined chromatographically after hydrolysis. The relative amounts of the different sugars present in the hydrolysates were determined by Somogyi's micro-copper method.[17] This Fehling's-acetone procedure may not lead to complete recovery of certain low-molecular polysaccharides such as galactans and arabans; such materials, however, also resist precipitation by ammonium sulphate and can thus be recovered from the mother liquor[12] of extracts which have been saturated with $(NH_4)_2SO_4$.

Aqueous extracts of germ and endosperm were therefore saturated with ammonium sulphate, and residual polysaccharides were recovered by acetone precipitation of the dialysed mother liquors and examined chromatographically after hydrolysis. The yields of the various products and their percentage compositions are shown in Table I.

TABLE I

CEREAL HEMICELLULOSES

Source and solubility characteristics of product	Yield (g. per 100 g. cereal preparation)	Composition (anhydro sugars, % of product): Glucose	Arabinose	Xylose	Galactose	Galacturonic acid
Pearl barley: water-soluble	0·92	74	10	16	—	—
Pearl barley: NaOH-soluble	2·46	77	8	15	—	—
Pearl barley: soluble in saturated $(NH_4)_2SO_4$	0·03	>95	*	*	—	—
Wheat germ: water-soluble	0·43	59	20	7	15	—
Wheat germ: NaOH-soluble	0·37	39	35	20	—	6
Wheat germ: soluble in saturated $(NH_4)_2SO_4$	0·18	15	38	4	43	—

* Traces of pentose on chromatogram: insufficient for estimation.

Where comparison with previous work is possible—*viz.*, yields of water-soluble fractions and yields and composition of soda-soluble endospermic material—it is found that the results obtained are in broad general agreement with those earlier analyses.[11,12,16] The products from wheat germ, which had not previously been examined in detail, were of interest in that a substantial amount of galactose was present in hydrolysates even of the crude Fehling's-acetone precipitate, while the mother liquor fraction from the $(NH_4)_2SO_4$ treatment contained over 40% of galactan. This is in marked contrast to the polysaccharides extracted from the endosperm, where no galactan could be detected. The NaOH-soluble hemicellulose from wheat germ was distinguished from all other products of this series by its content of galacturonic acid, which was present in sufficient concentration to be easily detected on paper chromatograms of hydrolysates.

A second series of extractions was designed specifically to prepare pectin from the two cereal products. Hirst *et al.*,[3] who have achieved signal success in separating galactan, araban and galacturonic acid from various seeds known to be rich in the different pectic components, used dilute boiling KOH to extract the pectin from de-proteinized material, and their methods were applied, with slight modifications, to the cereals. The general procedure adopted was as follows: Sugars and fats were removed by refluxing a finely-ground sample of the appropriate material in boiling 80% ethanol, and the residue was exhaustively extracted with 10% aqueous NaCl at room temperature and thoroughly washed with water. The turbid liquid removed by the salt treatment was discarded and the residue was extracted with boiling 0·2% KOH for three 2-hr. periods. After centrifugation, the bright extract was poured into 4 vol. of acid ethanol, the precipitate was re-suspended in water and reprecipitated and washed with 70% ethanol till free from chloride; it was then dried and weighed. With wheat germ this procedure gave manageable extracts and firm, slowly-forming precipitates, but with pearl barley removal of starch was essential before extraction with boiling alkali. This was accomplished by alternate boiling and treatment with malt α-amylase at 65° C. till neither the supernatant liquid nor the residual solid gave any reaction with iodine. The supernatant was then dialysed till free from dextrins and sugars and poured into four volumes of ethanol, the resulting precipitate being dried and examined in the usual way.

It should be emphasized that the malt amylase preparation used produced no low-molecular materials other than glucose and maltodextrins from a complex polysaccharide mixture of crude galactan, pentosan and starch; loss of pectin during this unavoidable enzymic treatment must therefore have been minimal and purely mechanical.

The precipitates were hydrolysed with N H_2SO_4 for 4 hr., the hydrolysates were neutralized with NaOH and, after removal of sodium sulphate, the products of hydrolysis were determined chromatographically. Results of these determinations are given in Table II, where it will be seen that the endospermic product closely resembles in composition the usual cereal hemicelluloses with no significant amounts of galactan or polygalacturonic acid and with a high xylan: araban ratio. The product from the embryo, however, is extremely heterogeneous. The presence of ribose in the hydrolysate indicates probable contamination with nucleic acids, despite the exhaustive preliminary extractions; the high arabinose : xylose ratio suggests the presence of a free araban, galactan is unequivocally present, and free galacturonic acid accounts for some 8% of the hydrolysate. The higher oligosaccharides referred to in Table II could not be characterized; they resisted prolonged hydrolysis with dilute acid and gave a strong uronic reaction after treatment with concentrated H_2SO_4 and carbazole. These partially hydrolysed products accounted for approximately 5% of the reducing activity of a hydrolysate but, in view of the unknown complexity of the individual components, no accurate assessment can be made of their total contribution to the hydrolysate. For the present purpose, however, this is not important; the significant finding is that a method which has proved outstandingly successful for characterizing pectin from several species of seeds has completely failed to reveal any pectin in pearl barley, while allowing unequivocal detection of the triad of pectic materials in material from the embryo.

The presence of ribose, and, therefore by implication, of nucleic acid, in the preparation from wheat germ is unfortunate in that it renders open to suspicion the determinations of calcium number and equivalent usually made for crude pectins. A further attempt was therefore made to obtain a purer sample of pectin, using ammonium oxalate as extractant. MacWilliam[8] has employed 50% acetone in pre-treatment of hops prior to oxalate extraction with considerable success, and his method was therefore applied to the cereal products. A sample of finely-ground wheat germ which had first been treated with boiling ethanol and then with water at 40° C. was exhaustively washed with 50% acetone prior to extraction with boiling water and then with 0·5% ammonium oxalate. Pectic materials were precipitated, dried and analysed by the usual methods, with the results shown in Table III. Once again, however, the pectin was very impure and attempts to purify it via the calcium salt were unsuccessful.

TABLE II

POLYSACCHARIDES EXTRACTED BY BOILING KOH

Hydrolysis products	Wheat germ		Pearl barley	
	% composition of product	mg. per 100 g. of cereal preparation	% composition of product	mg. per 100 g. of cereal preparation
Glucose	50	350	77	220
Arabinose	16	112	8	23
Xylose	6	42	15	43
Galactose	9	63	—	—
Galacturonic acid	8	56	—	—
Higher oligosaccharides	5	>35†	—	—
Ribose	6	42	—	—
Total yield*		700		286

* Corrected for non-carbohydrate impurities.

† Minimum value: no allowance made for degree of polymerization.

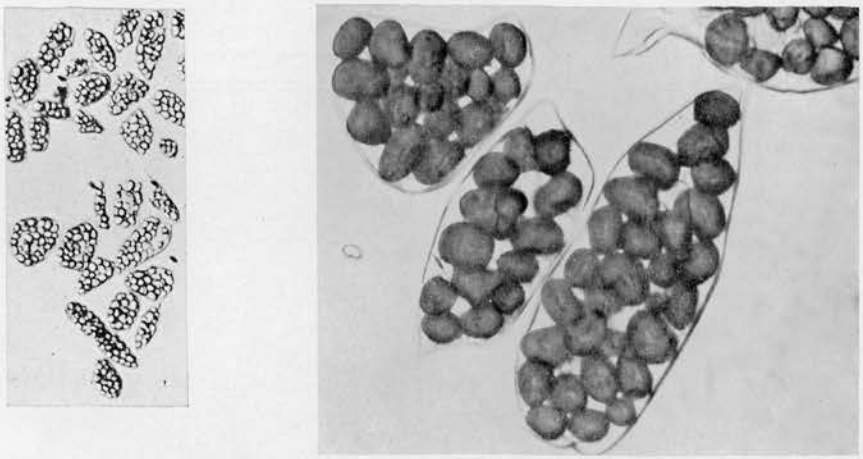

Fig. 1*a* and 1*b*.—Endosperm cells of *Bromus sterilis*, separating in water (1*a* × 60; 1*b* × 300).

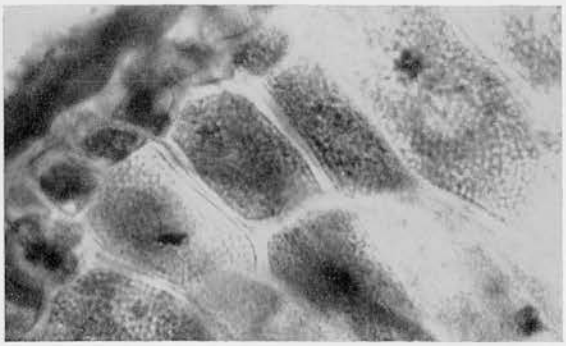

Fig. 2.—T.S. of endosperm of *B. mollis* in water; no cell separation (× 300).

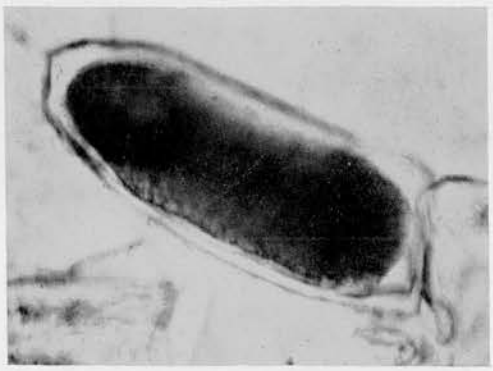

Fig. 3.—T.S. of endosperm of *B. mollis* in papain; cells separating with slight pressure (× 300).

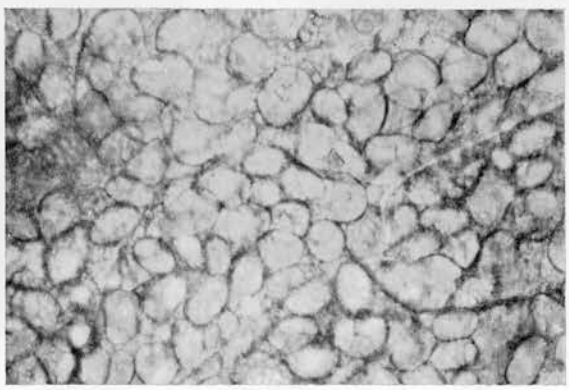

Fig. 4.—T.S. of barley endosperm in water; no cell separation (× 300).

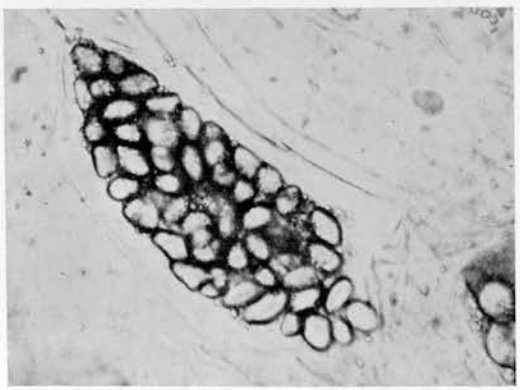

Fig. 5.—T.S. of barley endosperm in papain; cells separating with slight pressure (× 300).

Finally, with so many mixed products available, it seemed of interest to determine whether the postulated araban could be obtained as a separate entity. The evidence for pure araban in the embryo as distinct from the known xyloaraban of the endosperm

TABLE III

"PECTIN" FROM WHEAT GERM: PRE-TREATMENT WITH ACETONE

Hydrolysis products	Yield (mg. per 100 g. cereal product): Boiling water extract	Ammonium oxalate extract	Total
Glucose	49	170	219
Arabinose	32	77	109
Xylose	18	53	71
Galactose	29	40	69
Galacturonic acid	19	50	69
Higher oligosaccharides	†	†	†
Ribose	—	58	58
Total yield*	147	448	595

* Corrected for non-carbohydrate impurities.
† Present, but not estimated.

rests on the high arabinose : xylose ratio in hydrolysates of certain preparations from the germ; if free araban is present it should prove possible to separate some of it from concomitants by virtue of its greater solubility in 70% ethanol. To this end, 90 mg. of the mother-liquor fraction from the $(NH_4)_2SO_4$ treatment of an aqueous extract of wheat germ was shaken intermittently for 3 weeks in 70% ethanol and then separated by centrifugation. The ethanolic extract was concentrated, hydrolysed and examined; the hydrolysate contained 15 mg. sugar (as arabinose) and a chromatogram revealed arabinose as the only pentose, with a very faint smear in the glucose-galactose region. The ethanol-insoluble residue, on the other hand, still contained all the sugar residues present in the starting material, though with a marked diminution in the content of arabinose (Table IV). It seems, therefore, that an almost pure araban can be separated, in very small amounts, from wheat-germ preparations.

Histological studies.—While the chemical analyses reported above serve to strengthen the hypothesis that pectin is present in the embryo of cereal seeds though not detectable in the endosperm, they do not offer any evidence as to the nature of the material causing cohesion of individual endosperm cells. The behaviour in different media of thin sections of plant tissues can give some indication of the chemical nature of various cell-wall components, and an attempt was therefore made to determine the reactions of sections of endosperms in selected solvents. Although the present investigation has been specifically designed to consolidate and advance knowledge of cell-wall modification during the malting of barley, it was found useful in the first instance to examine not barley, but various species of the grass *Bromus*. Barley, with its very thin cell walls, gives sections whose cells rupture easily to release the enclosed starch grains, thus obscuring changes in wall structure, while *Bromus*, which strongly resembles barley in the nature of its extractable carbohydrates,[7] has a sturdy wall which is resistant to mechanical damage and easily observed under the microscope. Seeds of two species of *Bromus* were used, *B. sterilis* and *B. mollis*, and sections of the endospermic region were cut by hand, immersed in the selected medium in presence of thymol, and examined at intervals. At each examination, the material was mounted in the steeping medium under a cover glass, and, after direct observations had been recorded, slight rotatory pressure was applied to the cover glass and the material was re-examined.

TABLE IV

FRACTIONATION OF LOW-MOLECULAR POLYSACCHARIDE

Hydrolysis products	Amount in fraction (mg.): Starting material	Ethanol-soluble*	Ethanol-insoluble
Glucose	13·5	±	12
Arabinose	34	+++	20
Xylose	3·5	—	3·5
Galactose	39	±	34
Total	90	15	69·5

* +++, major constituent; ±, trace.

Of the enzyme preparations used (Table V) papain was a commercial product and "cytase" was a crude acetone-precipitated powder prepared from germinating *Bromus mollis*. The papain was apparently without action on β-glucan or on pentosan while the cytase rapidly reduced the viscosity of solutions of both these polysaccharides. Indeed

the cytase from *Bromus* showed higher pentosanase activity than did any other enzyme similarly prepared from raw or germinating cereals. With the results of this work with *Bromus* as a guide, similar tests were applied to sections of a number of different varieties of barley; results are shown in Table VI.

TABLE V

BEHAVIOUR IN DIFFERENT MEDIA OF SECTIONS OF ENDOSPERM OF *Bromus* SPP.

Medium	Reaction after 16 hr.	
	Bromus sterilis	*Bromus mollis*
Water	Complete separation of cells to individual units*	No separation, even after strong pressure†
0·5% $HgCl_2$	As in water	As in water
30% $(NH_4)_2SO_4$	No cell separation	No cell separation
0·1% papain	As in water	Separation of cells to blocks or to individual units‡
Cytase	Walls dissolved, leaving a thin membrane; starch grains emerging under pressure	Walls dissolved, leaving a thin membrane; the very small starch grains emerge easily

* Fig. 1. † Fig. 2. ‡ Fig. 3.

As can be seen from Table V, cells from the endosperm of *B. sterilis* showed no great tendency to cohere (Fig. 1, *a* and *b*) and only by mounting sections in solutions of ammonium sulphate was it possible to retain the individual cells of the endosperm in some semblance of a tissue. With *B. mollis*, however, there was no separation of individual cells in water (Fig. 2) and treatment with papain was necessary before separation could be achieved (Fig. 3). It is of interest to note that the cut end of *B. sterilis* was invariably white and mealy, whereas that of *B. mollis* was grey and glassy, recalling the appearance of typical steely barley. Of the barley samples examined (Table VI) those from the 1957 crop behaved like *B. mollis* (Figs. 4, 5) while two 1956 samples tended to behave like *B. sterilis*, with separation into blocks of cells or individual units after 16–24 hr. immersion in water. With barley, however, there did not appear to be a regular correlation between mealiness and ease of separation of cellular units of endosperm. Several samples each of *B. sterilis* and *B. mollis* were examined; the behaviour in each species remained constant.

DISCUSSION

Chemical analyses.—The determinations reported above (Tables I-IV) provide further confirmation of the virtual absence of pectic materials from the middle lamella region of endosperm cells of barley. There can be little doubt, however, that polymerized galacturonic acid, galactan and araban are all present in cereal embryos and that the middle lamellae of the germ are of the usual pectic nature.

It is becoming increasingly clear that analyses of whole grain may yield results very different from analyses of the separate constituent tissues—embryo, endosperm and husk. The embryo, rich in protein, in sucrose and in raffinose and apparently containing almost all the fat and pectin, differs so markedly from the endosperm, which contains all the starch and much of the β-glucan, that it is difficult to realize that both tissues originate from sister sets of gametes. The husk again differs from both embryo and endosperm in its virtual freedom from water-soluble carbohydrates and in its high content of hemicelluloses which resemble those of straws[12] and differ from the typical endospermic polysaccharides. Deductions made from gross analyses of whole grain may thus easily be misleading, as a substance which appears as a trace in such an analysis may well owe its apparent unimportance to localized distribution. Thus raffinose, for example, which accounts for less than 1% of the dry weight of the whole grain would

TABLE VI

BEHAVIOUR IN DIFFERENT MEDIA* OF SECTIONS OF BARLEY ENDOSPERM

Barley variety	Reaction
Ymer 1957 ..	Similar to *B. mollis*.†
Spratt-Archer 1957	Similar to *B. mollis*.
Earl 1957	Similar to *B. mollis*.
Proctor 1957 ..	Similar to *B. mollis* at periphery, with some separation in water at centre.
Ymer 1956 ..	Similar to *B. sterilis*, with cell separation beginning in centre.
Spratt-Archer 1956	Similar to *B. sterilis*, with cell separation beginning in centre.

* For media used, see Table V. † See Figs. 4 and 5.

appear to be of very minor significance—yet this trisaccharide represents over 10% of the mass of the embryo and is metabolized with extraordinary rapidity when growth begins. It is of interest to note that galactan (of wheat at least) is also predominant in the embryo; this co-existence of raffinose and galactan seems to be of rather general occurrence although as yet no clear relationship has been demonstrated between the galactose-containing trisaccharide and the polymer, galactan.

Pectin also, like raffinose, seems to owe its humble status as a grain constituent not to its being spread thin throughout the whole grain but rather to its being more or less restricted to the embryo, where it can be clearly demonstrated both by staining with ruthenium red and by chemical analysis.

Histological findings.—The rapid separation of cells from *B. sterilis* and from some samples of barley indicates that any intercellular material present must be easily water-soluble. It seems improbable that the cell separation is enzymically controlled as no inhibition of separation was observed in presence of mercuric chloride; in any event the enzymes in the ungerminated grain are primarily in the embryo and the sections under consideration contained no embryonic material. Dissolution under the influence of enzymes secreted by the living aleurone cells is also improbable, as cell separation regularly began in the centre of the section—the region where, it will be recalled, free nuclear formation was most pronounced in early stages of endosperm development. If there is indeed any material present between adjoining cell walls of *B. sterilis*—and it must be emphasized that there is no real evidence for the presence of anything tangible in this region—then such postulated material must be insoluble in ammonium sulphate (Table V). Such material could be, for example, protein or polysaccharide such as β-glucan, but it may well be that the action of this precipitant is to cause hardening of the actual external wall surface, with consequent intermingling of fibres from adjacent walls. Matters are less simple with *B. mollis* and with the majority of the barley samples examined. From the results with papain, it seems clear that a matrix of protein must exist between the cells—a matrix which hinders cell separation in water and which can be removed under the influence of proteolytic enzymes. The presence of such a matrix, impeding mechanical solution of normally water-soluble hemicelluloses, has already been suggested by Preece & MacDougall[15] and the observable behaviour of sections of certain samples of barley affords further confirmation of their suggestion. Although separation of individual cells was accomplished by proteolytic enzyme preparations, actual dissolution of the thick walls of the two species of *Bromus* was observed only in presence of cytase. After treatment with this enzyme packets of starch grains were apparent, each packet surrounded by a very thin membrane whose chemical nature is as yet unknown, though it appears to correspond to the "cellulose" reputed to persist in malt corns.

General.—When the results discussed above are considered in relation to cell-wall modification during malting, it is clear that they provide further confirmation of a number of well-established principles. Thus, dissolution of pectic substances is truly of no consequence whatever—as no pectic material is present in the endosperm to impede solubilization. Secondly, the traditional dislike of high-nitrogen barley for malting may well be founded on the possible *location* of the excess protein; if this is indeed present in the middle-lamella region, as the preliminary results discussed above would suggest, its first effect would be to prevent solubilization—mechanical or enzymatic—of the hemicelluloses of the cell wall. It has already been suggested that papain may act in barley extracts to unmask some of the hemicelluloses,[15] thus making possible higher yields of, for example, water-soluble β-glucan; such a masking effect of protein would be expected to be more pronounced in intact germinating grain where physical cohesion of cells would render ingress of solvents even more difficult.

Over the past decade considerable advances have been made in understanding the changes which take place in the carbohydrates of the endosperm; the time is now surely ripe for a further study of the origin and dissolution of nitrogenous compounds, not merely in the whole grain, but specifically in the endosperm. The results of such a study, taken in conjunction with what is now known about polysaccharide degradation, might well be of far-reaching practical importance.

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VII

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CELLULOSE DISTRIBUTION IN BARLEY

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A crude cellulose, prepared from Proctor barley after mild acid hydrolysis, accounted for 4·37% of the dry weight of the grain, and contained approximately 8% of xylose residues and 20% of mannose residues in addition to glucan. Mannan was not detected in preparations from barley embryos. Almost all of the cellulose was restricted to the outer rubbings of the grain with only 1/25 of the total in the final pearl which itself represented nearly half of the grain weight. The cellulose from the pearled grain resembled that from the husk both chemically and microscopically, and the amount of tissue in the furrow would suffice to account for all of the cellulose in the pearl. It is suggested that the cell walls of the starchy endosperm may be free from cellulose; consequently, a true cellulase is not of any importance in malting.

Introduction

In 1890, Brown & Morris[4] published the first reasonably complete description of the microscopically-detectable changes undergone by barley in the course of its transformation to malt, and for meticulous observation of the germinating grain this account has almost certainly never been bettered. Brown & Morris were especially interested in the dissolution of endosperm cell walls under the influence of a secretion from the scutellar epithelium, a secretion which they described as a "cytohydrolytic enzyme" acting on the "cellulose" forming these walls. At the time their paper was published, the terms "cellulose" and "unlignified cell wall" were virtually synonymous, though it was beginning to be recognized that certain seeds contained in their cell walls a rather labile carbohydrate fraction which was sometimes distinguished under the name of "reserve cellulose." Many of the so-called reserve celluloses would now be classed as hemicelluloses, soluble in dilute alkali and containing a proportion of

β-linked sugar residues other than glucose; but in 1890 work on hemicellulose analysis had hardly begun, and solubilization of a cell wall was accepted as evidence for solubilization of cellulose.

Brown & Morris were able to show that crude enzyme preparations from low-dried malt were able to dissolve barley endosperm cell walls, first causing swelling of the different layers and then later partially degrading the solid material to leave a ghost-like wall composed of numerous spindle-shaped fragments which persisted for some time after the wall had really ceased to be a continuous structure. Other observers, notably Mann & Harlan,[15] considered that the ghost wall detectable in malt was a more solid layer which could be clearly differentiated by staining with congo red.

While deferring for the moment any further consideration of the residual cell wall of malt endosperm, it is of interest to consider the possible nature of the material which is unequivocally removed from the walls under the influence of barley enzymes. Horace Brown himself must certainly have appreciated the potential over-simplification involved in referring to the endosperm walls as cellulose, for, in a later paper,[3] he consistently omits reference to cellulose and uses in its place the less specific but wholly accurate term "cell wall." Other writers have not all been so cautious, and in two recent discussions of barley composition, selected at random, the mutually-exclusive statements that "the cell walls are composed of hemicellulose united by a network of nitrogenous and gummy material"[5] and . . . "the principal component of the cell wall, namely cellulose . . . ,"[7] are made without qualification. Hemicelluloses from barley endosperm are well authenticated,[18] and the apparent ubiquity of cellulose in angiosperm cells makes it at least highly probable that some cellulose is present —though it should be remembered that the deposition of endosperm walls in grasses follows rather an unusual course.[13] Unfortunately there do not seem to be any histological tests which distinguish unequivocally between cellulose and hemicellulose, and recourse must be had to rather tedious methods of chemical analysis for satisfactory differentiation of the two materials. Such analyses do not seem to have been attempted for homogeneous samples of barley endosperm though, in spite of the difficulties involved, the results would be of considerable interest both from a practical and from a more academic point of view. Certainly, until such time as the initial composition of the endosperm wall is precisely known, speculations regarding the nature of the material removed from it during malting must inevitably be limited.

Practical implications.—One desideratum in a malting barley is that the cell walls of the endosperm should be readily solubilized without undue loss of valuable sugar residues to respiration or to synthesis of new seedling material. Attention has recently been directed to the possibility of controlling malting loss by minimizing respiration or by arresting seedling growth,[10] but there is still considerable interest in the complementary approach—that of achieving optimum modification by selecting barleys with the best balance of cell-wall material and enzymes capable of degrading these cell walls. Methods adopted for measuring cell-wall degrading potentialities have included autolytic techniques which take cognizance of both the level of hemicellulase activity and the amount of readily solubilized substrate in the ungerminated grain,[17] and have included also determinations of hydrolytic potentialities of barley extracts towards substituted celluloses such as ethyl-hydroxyethyl-cellulose.[20] The autolysis method certainly measures the breakdown of a carbohydrate mixture which is actually present in barley endosperm, though the distribution of this carbohydrate material within the cells is not known, and its possible effects in impeding modification are therefore rather obscure. On the other hand, though the possession of enzymic potentialities for cleavage of a substituted cellulose may correlate highly with the presence of enzymes actually involved in wall lysis, complete reliance on observations made with an artificial substrate may on occasion give misleading results. It would appear to be highly desirable, therefore, to obtain precise figures for the cellulose content of endosperm cell walls as a preliminary to deciding whether estimates of cellulase in germinating barley are of genuine importance. Estimates are certainly available for the cellulose contents of several different varieties of barley, but the relative proportions of the total cellulose to be assigned to husk, embryo and endosperm do not seem to have been ascertained, and, in relation to modification, it is

only the possible cellulose in the cell walls of the endosperm which is of importance.

Theoretical considerations.—The more academic implications of the structure of the endosperm cell wall are also of very considerable interest. Very many review articles dealing with cellulose decomposition describe germinating barley as their only example of an angiosperm capable of producing a true cellulase, and, among a galaxy of well-authenticated fungal and molluscan enzymes, barley "cellulase" is listed as a well-known cellulose-decomposing enzyme from a seed plant. As review succeeds review, possible references to "barley cellulase" accumulate, but, as far as we have been able to ascertain, all these references are ultimately based on a very small number of original research papers. Typical of experimental results quoted as providing evidence for the presence of cellulase in germinating barley is the work of Goerdeler,[6] which is worth considering in some detail. Using as substrate a type of cellulose dextrin prepared by treating filter paper with 62·5% sulphuric acid at room temperature for 5 hr., Goerdeler showed that crude enzyme preparations from barley, acting in rather massive doses, were able to induce some formation of dextrorotatory material after incubation for 6–7 days at 30° C. There was a slight enhancement of enzymic activity on germination, but neither barley enzymes nor enzymes from germinated grain were capable of any attack whatsoever on untreated filter paper.

The evidence for a barley cellulase, active during malting, thus seems to rest principally on the observation that endosperm cell walls, of uncertain composition, are partly solubilized under the influence of scutellar secretions, and on the demonstration that extracts of barley can partially hydrolyse degraded cellulose at an extremely slow rate. Such evidence hardly seems to justify the segregation of germinating barley from all other seed plants with respect to its cellulase-producing potentialities, especially when barley extracts are apparently devoid of action on undegraded cellulose.

The nature of the cell wall.—Although cellulose itself can be precisely defined as a long-chain fibrous polymer of β-1·4-linked glucose residues, this exact chemical knowledge of its structure is of no great assistance in carrying out determinations of cellulose in plant tissues, since various other carbohydrates, found in more or less close association with cellulose, introduce considerable difficulties in purification. Most intimately associated with cellulose is the so-called cellulosan which at least partly resists acid and alkaline extraction and which, on analysis, is frequently found to contain substantial amounts of xylan or mannan. The polysaccharides more loosely associated with the cellulose of the wall are often referred to as encrusting hemicelluloses; on hydrolysis they yield a wide variety of sugars units and, frequently, uronic acids. The adjective "encrusting" rather implies that these hemicelluloses form some sort of surface skin on the underlying cellulose, but there is no evidence to indicate the actual position of encrusting hemicellulose in the cell wall, so the term "encrusting" is possibly potentially misleading. This encrusting hemicellulose is often associated with lignin, and methods designed to remove lignin from cellulose also remove at least a part of the encrusting hemicelluloses. Finally there are the polyoses—glucans and pentosans—which may be directly water-soluble or capable of being rendered water-soluble by enzymic depolymerization. Certain of these polyoses have now been characterized chemically, and the most fully investigated, β-glucan of barley, has been found to resemble lichenin rather than cellulose.[1]

With this wide variety of components present in a plant cell wall, and with precise chemical definition of structure available only for the least soluble and for one of the most soluble members of the group, it is not surprising that knowledge of cell wall composition and metabolism is still rather imperfect. However, methods are available for achieving considerable purification of the more resistant fractions of the wall and so providing a measure of a rather empirically-secured structural carbohydrate residue, such as "crude cellulose" or "insoluble carbohydrate." The crude cellulose determination of Cross & Bevan, as modified by Norman & Jenkins[16] entails removal of lignin by treatment with acid hypochlorite and subsequent dissolution of the "lignone chloride" in boiling sulphite. Crude cellulose from 2-rowed barley amounts to approximately 6% of the dry weight; after correction for pentosan remaining in the crude cellulose, a figure of approximately 5% of "true" cellulose is obtained. Estimations of "insoluble

carbohydrate" as carried out by Bishop & Marx[2] can be performed more rapidly than can analyses for crude cellulose, and as long as the fineness of grind and the times of acid and alkaline hydrolysis are strictly controlled, results are reproducible. For 2-rowed barleys figures for "insoluble carbohydrate" average 7% of the dry weight of the grain. These figures all refer to the whole corn; analyses of husk, which comprises some 11% of the grain, indicate that the husk contains about 24% of cellulose,[12] thus leaving approximately 2·4% to partition among the meristematic embryo cells, the thick-walled aleurone and the central starchy endosperm. These figures are all necessarily only approximations, as they have been assembled from results of several different workers using different barley varieties and different techniques; the following analyses provide comparable data for an arbitrarily-defined cellulose fraction from different layers of the grains of one variety of barley, analysed by identical methods throughout.

Experimental

Preparation of crude cellulose.—A sample of Proctor barley from the 1957 harvest was treated in an experimental pearling machine to yield 6 fractions successively representing progressively deeper layers of the grain. Although the resulting individual fractions were not botanically homogeneous, the major constituent of each could be identified microscopically (Table I) and the treatment gave a reasonably satisfactory separation into husk (1st rubbings), embryo plus aleurone (2nd–4th rubbings), and starchy endosperm (5th rubbings and pearl). Owing to the asymmetric nature of the corn, with its deeply incised furrow, traces of husk persisted even in the final pearl. The proportionate frequency of husk fragments present in the three final fractions was determined by staining with crystal violet and decolorizing in NaOH, as recommended by Larkin *et al.*[11]

TABLE I

Barley Fractionation

Fraction	% of whole grain	Macroscopic and microscopic appearance
1st rubbings	10·92	Mainly large pieces of husk with a few fragments of rootlets and aleurone and a light sprinkling of starch grains.
2nd rubbings	4·24	Light brown and containing small husk fragments, some entire embryos and aleurone. Starch from broken endosperm cells present.
3rd rubbings	9·41	Very light brown and containing, in addition to aleurone, embryo fragments and starch grains, some whole starchy endosperm cells. Husk fragments too numerous to count.*
4th rubbings	13·81	Cream coloured. Many starch grains and a little aleurone present and portions of endosperm walls, rendered visible by congo red staining; no embryo tissues detected. 26 husk fragments per microscope field.*
5th rubbings	13·56	Almost white in colour, but otherwise resembling 4th rubbings. 9 husk fragments per microscope field.*
Pearl	46·71	Surface of starchy endosperm exposed except in furrow, where husk, chalaza and aleurone were still present. 5 husk fragments per microscope field.*

* Method of Larkin *et al.*[11]

Initially it was hoped to prepare samples of Cross & Bevan cellulose from the whole grain and from the constituent fractions, but with the more starchy material filtration was so slow even after prolonged pre-treatment with amylase that the standard method was abandoned and all fractions were subjected to a preliminary mild hydrolysis with hot dilute sulphuric acid. Such treatment cannot affect true cellulose, but it will hydrolyse some of the encrusting hemicellulose and may remove a proportion of the cellulosan. The method of preparation finally adopted, which proved satisfactorily reproducible in that results by two separate workers agreed to within 2%, was as follows: a suitable amount of ground barley or barley fraction was suspended in N H_2SO_4 and boiled under reflux

for precisely 4 hr., with careful agitation during the first 30 min. to prevent charring. The contents of the flask were then centrifuged, washed twice with hot water and suspended in 100 ml. of water at room temperature. 5 ml. of commercial sodium hypochlorite solution and 2 ml. of 0·2-N H_2SO_4 were added and the mixture was stirred for 10 min. After filtration on a tared No. 2 sintered glass crucible, the residue was washed with hot water and returned to the beaker. The "lignone chloride" which had formed was then extracted by boiling in 6% sodium sulphite for 20 min., and the residue was again filtered and washed. After 5 successive chlorinations and sulphite extractions the residue from the whole grain was white and fluffy and was adjudged to be free from lignin; with the pearl barley the product appeared almost white after only 3 chlorinations but, in order to obtain fully comparable results, it also was subjected to 5 treatments. Finally the crude cellulose fractions were boiled for 2 min. with distilled water, washed, filtered, and washed on the filter twice with hot water and then successively with hot ethanol, cold petroleum ether and cold ethanol, and dried in a hot-air oven to constant weight. Losses throughout were minimized by using the same beaker and sintered glass crucible for all operations.

Preliminary work had shown that while 2 g. of husk sufficed to give an excellent yield of crude cellulose, 10 g. of pearl gave insufficient residual material for subsequent analyses, and approximately 25 g. of pearl was required to give a reasonable return. For the intermediate fractions 5–10 g. of starting material was used. All the dry products were white or very pale cream in colour and all were highly fibrous. Microscopic examination revealed the presence of two distinct elements—straight pitted fibres resembling delignified sclerenchyma, and corrugated material apparently derived from typical grass epidermal cells. A little amorphous material was also present. Yields, expressed as percentages of each starting material and also as proportionate contributions to the whole grain, are shown in Table II.

Analysis of crude cellulose.—From each product a 40-mg. sample of finely-divided material was suspended in 2 ml. of 72% (v/v) H_2SO_4 at room temperature. The fibres gradually swelled, turned yellow and then brown and finally formed a highly viscous dark solution. When solution appeared to be complete, the partly-hydrolysed material was poured into 30 ml. of water and boiled under reflux for 4 hr. to complete hydrolysis to the constituent sugar units. In preliminary work with husk preparations it was found that the frequently-recommended 24-hr. treatment with concentrated acid was not sufficient to allow complete hydrolysis on subsequent dilution and boiling, as only 60% of the expected monosaccharide was found in the hydrolysate and white flocs persisted even after prolonged boiling. This flocculent residue, collected by centrifugation and exhaustively washed with hot water, gave a strong carbohydrate reaction with anthrone and thus appeared to contain a considerable proportion of unhydrolysed cellulose. Use of more concentrated

TABLE II

YIELDS OF CRUDE CELLULOSE FROM BARLEY FRACTIONS

Fraction	Yield (%, dry weight)	Contribution to whole barley (g. per 100 g.)	% of total cellulose in fraction
1st rubbings	27·1	2·96	69
2nd rubbings	9·8	0·42	10
3rd rubbings	4·0	0·38	9
4th rubbings	1·9	0·26	6
5th rubbings	0·6	0·08	2
Pearl	0·4	0·19	4
Total	—	4·29	—
Unfractionated grain	4·37	4·37	—

acid or of higher temperatures tended to cause charring, but a 72-hr. exposure to 72% acid gave adequate preliminary solubilization of the cellulose from the husk. This length of pre-treatment was used for all fractions, but the percentage of reducing sugars in hydrolysates of material from the inner portions of the grain was very much less than theoretical (Table III). Again the residual unhydrolysed material appeared to contain carbohydrate and it seems that a completely reliable method of hydrolysis has not yet been secured. The fractions from the inner rubbings may have been contaminated with protein, but unfortunately there was not enough crude cellulose available from the more floury rubbings to allow exploration of other methods of hydrolysis.

crude cellulose. 200 mg. of the preparation from the husk were suspended in 20 ml. of 10% NaOH, left with occasional stirring for 24 hr., and then separated into soluble fraction and residue. When the residue was washed, dried and weighed, 20% of the original crude cellulose was found to have dissolved in the alkali. The alkaline extract on acidification gave a powdery precipitate which was collected, washed and suspended in 72% H_2SO_4, in which it appeared to dissolve almost instantaneously. After standing for 18 hr., the acid solution was diluted and boiled. Once again white flocs appeared on dilution; so, apparently, even the alkali-soluble fraction of crude cellulose of barley is remarkably resistant to acid hydrolysis. Chromatographic examination showed that

TABLE III

Composition of Crude Cellulose from Different Fractions

Fraction	Hydrolysis achieved (% of theoretical)	Sugar units (% on hydrolysate)		
		Glucose	Mannose	Xylose
1st rubbings	93	70	22	8
2nd rubbings	75	71	20	9
3rd rubbings	79	65	23	12
4th rubbings	70	74	19	7
5th rubbings	60	73	18	9
Pearl	60	65	27	8

After the reducing sugar content had been determined, the remainder of the hydrolysate was analysed chromatographically, using methods previously described,[19] with the results shown in Table III. The recognition of glucose and a small quantity of xylose in all hydrolysates was not unexpected, but the regular appearance of an aldohexose with the same mobility as mannose in butanol-acetic acid-water was rather surprising. When material from the mannose region of an unsprayed chromatogram was eluted and re-run in a number of solvents, the sugar consistently behaved in exactly the same manner as authentic mannose and, although the mannose from cellulose has not been characterized chemically, there seems no reason to doubt the validity of its chromatographic identification.

Since mannan from ivory-nut can apparently be extracted as a pure polysaccharide by solution in alkali, attempts were made to separate the mannan from barley the alkali-soluble material contained the same sugars as the original crude cellulose, namely glucose, mannose and xylose, but no attempt was made to determine the proportionate composition of the extracted carbohydrate.

As all the grain rubbings and the pearl contained at least traces of husk, and as husk contains a high proportion of cellulose, it was considered desirable to examine the cellulose from one husk-free fraction of the grain. Endosperm cannot easily be obtained free from husk, but embryos can be separated from grain which has been steeped for 2 hr. 200 embryos were excised, extracted with boiling ethanol, dried and hydrolysed for 4 hr. in N H_2SO_4. The residual material was thoroughly washed, treated with 72% H_2SO_4, hydrolysed and examined chromatographically. Chlorination and sulphite extraction were omitted, as the amount of impure fibrous material was less than 150 mg., and no attempt was made to estimate the sugar

content of the hydrolysate, as results could not be compared with those from other crude celluloses. Chromatograms of the hydrolysate revealed the presence of only glucose and a very little xylose, no trace of mannose being detected.

Discussion

Owing to the failure to secure complete hydrolysis of all the preparations of crude cellulose, it is not yet possible to come to any final conclusions regarding the chemical composition of the fractions prepared from different parts of the grain. The results shown in Table III do not, however, indicate any major difference between the structure of the crude cellulose from the husk and that from the final pearled grain. The material prepared from the 3rd rubbings, which contained a high proportion of embryo and aleurone, differed slightly, though possibly not significantly, from the other preparations in containing a rather higher proportion of xylose residues, but until a more satisfactory means of hydrolysis is achieved discussion of such slight differences must be unprofitable.

The crude cellulose from this sample of Proctor barley contained approximately 8% of xylose residues and, if the convention of computing "true cellulose" by applying a correction for pentosan content to the crude cellulose is followed, then the "true cellulose" is approximately 4% of the dry weight of the grain. This figure is rather low—5% is usually quoted for 2-rowed barleys—but the smaller husk content of Proctor barley[9] together with the fairly drastic preliminary treatment in acid would account for the lower figures reported here. That the acid pre-treatment does reduce yields is clear from a comparison of Cross & Bevan cellulose, prepared from whole grain in preliminary experiments, and crude cellulose prepared by the methods of the present investigation (Table IV). The Cross & Bevan cellulose lost some 20% of its sugar residues after 3 hr. hydrolysis with N H_2SO_4 and the unhydrolysed residue compared well, both in amount and in composition, with the crude cellulose prepared by the present technique.

Clearly, however, the figures quoted in Table IV for "true cellulose" do not represent exclusively β-1·4-glucan, as some 20% of mannose was present in the hydrolysates of all samples of crude cellulose. It will be recalled that Preece & Mackenzie[19] reported traces of mannan in water-soluble hemicellulose preparations from Pioneer barley, and substantial amounts of mannan have also been detected in preparations from certain wild grasses,[14] so the recognition of mannan in barley cellulose is not altogether surprising. Preliminary attempts to separate mannan from the glucan were not successful, and no useful comment can as yet be made regarding the association of mannan and cellulose in the cell wall. It is interesting to note, however, that a mannanase has been recorded from barley, and Luers[12] has suggested, in a teleological argument which, he acknowledges, is not without danger, that, where the enzyme is, there should the appropriate substrate be also. Whether the mannan now recorded from barley actually forms a potential substrate for mannanase during germination must depend, *inter alia*, on the location of both enzyme and polysaccharide within the grain, but further studies on the enzyme-substrate system might be of interest.

When a further correction is made for the mannan content of barley cellulose a final

TABLE IV

Comparison of Cross & Bevan Cellulose and Crude Cellulose of this Investigation

	Yield (%, dry barley)	Xylose in product		"True" Cellulose
		g.	%	
Cross & Bevan Cellulose:				
Total	5·58	1·126	22	4·45
Removed by acid hydrolysis	1·34	0·777	58	—
Residue	4·24	0·349	8	3·89
Present method	4·37	0·393	9	3·98

figure is obtained of 3 g. of resistant glucan per 100 g. of dry barley. This figure can presumably be taken as genuine true cellulose, and, of it, approximately 2 g. is contained in the outer 11% of the grain and only 0·12 g. in the whole of the inner 47% of the grain, *i.e.*, a mere 4% of an initially small amount of true cellulose is allotted to the central half of the corn. As was mentioned earlier, the furrow is still obvious in the pearl, and transverse sections show the typical sheaf cells leading from the chalaza to the endosperm. It will be recalled that the chalaza is derived from the stalk of the ovule and, genetically at least, it is comparable with the husk rather than with either embryo or endosperm. During the early stages of preparation of the crude cellulose from the pearl, small crescentic brown fragments were observed among the amorphous white material, and it seems highly probable that these fragments were derived from the furrow tissues of the pearl. Although no accurate quantitative assessment could be made of the contribution of cell walls of chalaza and husk to the pearl, measurements made on transverse and longitudinal sections through the furrow suggested that the material in the furrow represented approximately one-seventieth of the total volume of the pearl. If the composition of this extra-endospermic material is similar to that of the husk then it is sufficient to account for a yield of 0·39% of cellulose from the pearl; the yield actually recorded was 0·40%. Again, treatment of the different fractions with crystal violet suggested that the number of husk fragments in the flour from the pearl was roughly one-fifth of that from the 4th rubbings and, though this may be wholly coincidental, the percentage yield of cellulose from the pearl was also about one-fifth of that from the 4th rubbings (Table II).

Microscopic examination of the final products showed that the greater part of the crude cellulose from the pearl was derived from sclerenchyma fibres and from epidermal walls—cell types which are absent from the starchy endosperm, though present in the furrow tissue.

Taking the chemical and the anatomical evidence together, then, it may be definitely stated that the crude cellulose from pearl barley is virtually identical, chemically and microscopically, with that prepared from barley husk, that the total crude cellulose of the pearl can be accounted for by the extra-endospermic material trapped in the furrow, and that there is, therefore, no real evidence for the presence of any true cellulose in the central endosperm.

Reference was made in the Introduction to the ghost walls of malt endosperm; these structures can be stained with congo red and they are frequently regarded as the residual true cellulose of the walls. However, congo red is an acid diazo compound which is directly attached to cellulose molecules by forming hydrogen bonds with the hydroxyl groups; it cannot be regarded as diagnostic of cellulose, as certain extracted hemicelluloses stain brilliantly and persistently with congo red. Despite its reaction with a typical cellulose stain, the ghost wall of malt may easily be of resistant hemicellulose rather than of true cellulose.

So far in this Discussion reference has been made only to the husk and to the central endosperm, and the contribution of embryo, pericarp-testa and endosperm have been ignored. The embryo accounts for 2–3% of the grain, and a preliminary study of isolated embryos has suggested that the cellulose here differs from that of the lignified husk in lacking mannan. In seed plants generally, mannan tends to be found in lignified cell walls or in tuberous storage organs, and the absence of mannan from a meristem is to be expected. The total cellulose of the isolated embryos was not determined in the present study; James[8] has recorded a "cellulose" content of 3% of the dry weight of the embryos but this figure may be rather low, as, in his work, the cellulose was solubilized by exposure to 72% acid for only 3 hr.—a length of time which would now appear to be inadequate. The pericarp-testa is quantitatively insignificant and is remarkable only for the thick cuticle of the testa which almost completely obscures the cellular origin of that region. The aleurone cells, which are very thick-walled, may make some contribution to the cellulose of the grain. The nature of these walls does not seem to have been fully investigated, but it is noteworthy that, in sections of barley corns, these aleurone walls at least partially survived 30 min. boiling in N H_2SO_4 and subsequent immersion in N NaOH—a treatment which sufficed to solubilize all the starchy endosperm walls. The aleurone cell walls, incidentally, survive the malting process

substantially unchanged as far as can be detected by microscopic examination.

The position seems to be, then, that the husk, the embryo and the aleurone can together account for all the cellulose of a barley corn, with no significant contribution from the starchy endosperm cells. If this is true, and though some of the evidence is circumstantial there seems to be no reason to doubt it, then measurements of cellulase activity, carried out to assess the potential ease of modification of a barley sample, would seem to be rather ill-directed. Furthermore, claims for cellulase activity in germinating barley, which were initially based on the accurate observation that the endosperm walls are solubilized under the influence of an enzyme, should be critically scrutinized. Cellulase may be present, even although there is no cellulose to act as substrate, but such evidence as there is for an active cellulase is not very impressive, and is certainly insufficient to justify the segregation of barley from other seed plants as a source of cellulase.

Even if true cellulose is virtually absent from the endosperm, the walls as seen in sections of the corn are fairly robust structures, and there can be little doubt that hemicelluloses contribute significantly to their composition. The insoluble hemicelluloses have been rather neglected in recent years, and a fuller understanding of cell-wall modification must now surely depend on a more intimate knowledge of the structure of these hemicelluloses and of the potentialities of germinating grain for their solubilization.

Conclusion

This paper opened with a brief reference to the monumental work of Brown & Morris on barley germination. It is perhaps appropriate, therefore, that the principal conclusion to be drawn from a consideration of the results presented here should have been reached by Horace Brown[3] in 1916. Referring to the urgent need to understand the changes undergone by barley in its transformation to malt, Brown stated that

> "no real progress can be made in such an investigation as long as we adhere to the old plan of analysing the grain as a whole, before and after germination. The grain of barley is highly differentiated into various tissues and organs, and to deal with it in a wholesale way is about as reasonable as it would be to analyse a whole animal when investigating its life changes."

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VIII

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STUDIES ON THE FREE SUGARS OF THE BARLEY GRAIN—V. COMPARISON OF SUGARS AND FRUCTOSANS WITH THOSE OF OTHER CEREALS

BY

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V. COMPARISON OF SUGARS AND FRUCTOSANS WITH THOSE OF OTHER CEREALS

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Ripe intact grains of rye, wheat and barley are rich in fructosan and in glucodifructose, oats may contain moderate amounts of these carbohydrates, but maize contains neither. Fructosan content runs parallel with the content of water-soluble gum-like pentosans, so that the amounts of each decrease in the order: rye, wheat, barley, oats, maize. Moreover, the fructosan-rich and pentosan-rich cereals are those which contain abundant β-amylase in the raw grain. Taxonomic relationships of these aspects of parallel distribution of apparently unrelated materials are discussed, and it is suggested that the composition of the ripe grain depends on the degree of development along particular paths reached at the stage adjudged to represent ripeness, development differing to a major extent from one cereal to another and to a minor extent between samples of the same cereal, but depending also in some measure on post-harvest changes. Cereal adjuncts used for brewing contribute types and amounts of carbohydrates which depend on the nature of the raw cereal and on whether whole grain or separated endosperm is used in preparing the adjunct. Implications of the possible contribution of raffinose by certain adjuncts are discussed.

Introduction

In Parts I–IV of this investigation,[14,15,16,17] some detailed information has been presented in respect of the free sugars and oligosaccharides of barley, and their behaviour during malting has been described. As was emphasized earlier, the part played by the free sugars in initiating seedling growth, and in providing a substrate for respiration and synthesis during the early stages of establishment of the young plant, cannot wholly be separated from the influence of the more complex carbohydrates or from the effects of the enzyme complement of the grain; now that a reasonably complete account of the behaviour of individual sugars has been given,[17] it seems desirable to attempt to relate these observed changes in the low-molecular carbohydrate fractions to other changes known to occur during germination. Study of the available literature, however, reveals that, apart from the considerable volume of work on starch and on the amylases and the recent sustained interest in the high-molecular gums,[22,23,24,25,26,27] information about barley carbohydrates and, more especially, about the enzymes concerned in their synthesis and degradation, is disappointingly meagre.

Even if a comprehensive account of the individual carbohydrates of barley were available, difficulties of interpretation of data would still remain; too intensive interest in one particular species of plant may defeat its own ends, as from a narrowly restricted range of facts it is only too easy to draw highly plausible but wholly erroneous conclusions. By broadening the basis of the present study to include a number of different cereal grains, it was hoped at least partially to avoid the danger of over-confident deduction from a necessarily limited range of results. Again, by establishing the carbohydrate balance of the ungerminated grains of a number of more or less closely related cereals, and by considering the sugar status in relation to other factors where these have already been studied, it should be possible to focus attention on the most obvious gaps in present knowledge, and so to select the topics most likely to yield rewarding information in further extension of the study of possible metabolic changes during germination. The inclusion of other cereal grains in a study specifically devoted to barley is, therefore, not simply a means of extending present knowledge of the chemistry of individual cereals—desirable though such extension might be—but rather is intended to provide reference data for comparative study, with barley and the behaviour of its sugars and oligosaccharides remaining the central object of interest.

The cereals chosen for comparative examination were wheat (*Triticum sativum* Lam.), rye (*Secale cereale* Linn.), oats (*Avena sativa* Linn.), and dent maize (*Zea mays* Linn.). Broadly speaking, these cereals represent two of the four major taxonomic groups of the Gramineae, and, though their classification is based primarily on the structure and arrangement of the spikelets, a number of subsidiary points are of some diagnostic value.[12] Thus maize, a typical member of the tribe Maydeae, is one of a group of tropical or sub-tropical grasses, all with small chromosomes, with n based on 5, 9 or 12, and all having a panicoid type of leaf anatomy. Rye, wheat and oats are grasses of temperate climates, with large chromosomes, n based on 7, and a festucoid type of leaf anatomy. Along with barley, rye and wheat are placed in the tribe Hordeae, all members of which have large, oval, simple starch grains, whereas oats (Avenaeae) has small polygonal starch grains united into aggregates in a manner reminiscent of those of rice.[30] In addition to the details of floral morphology—the criteria most frequently used in taxonomy—it is clear that anatomical and genetical aspects of structure may be at least of corroborative value.

Within the tribe Hordeae, wheat, rye and *Agropyron* (*e.g.*, couch grass) are usually assigned to the Triticineae, and the hybrids obtainable (wheat × rye) are indicative of the rather close relationships subsisting amongst the members of this group. Barley and *Elymus* are classed in Elymineae, and though spikelet arrangements may warrant this distinction, the fact that an artificial hybrid from *Agropyron* × *Elymus* has been produced,[9] and a probably natural hybrid of *Agropyron* × barley described,[12] suggests that the sub-division of the tribe may be rather an artificial one designed for the convenience of taxonomists.

According to Belval,[2] the cultivated cereals rye, wheat and barley resemble one another in containing the fructosan levosin, which is fairly readily attacked by yeast invertase, whereas certain other members of the

Hordeae (*e.g.*, *Agropyron repens*, *Elymus arenarius* and *Hordeum bulbosum*) contain a fructosan resistant to invertase. Although chemically-distinct polysaccharides may indeed be concerned, it must be remembered that in barley, and probably in certain other cereals also, "fructosan" is not a single, well-defined chemical entity but rather represents a series of oligosaccharides characterized by possessing increasing numbers of fructose residues with a single terminal glucose group; the lower members of the series are readily hydrolysed by invertase, whereas the higher-molecular materials are resistant to that enzyme.[16] It is possible that the fructosans typical of certain species may normally exhibit a slightly greater chain length in their ultimate members, with, consequently, a lower proportion of glucose, a more negative specific rotation, and a lessened susceptibility to invertase action. Though attempts have been made to relate the classification of the Hordeae to the nature of their fructosans,[7] it would be wiser to defer further consideration of this question until a comprehensive survey has been made of the types of linkage in the molecules.

Over a more general field, however, chemical affinities in the Gramineae are rather closely related to taxonomic data. Thus, de Cugnac[8] has divided grasses into two distinct groups: those which form fructosans (wheat, barley, oats and rye, as well as many temperate wild grasses) and those which do not at any time form fructosans (maize, sugar cane, rice and sorghum, together with a heterogeneous assortment of wild grasses). At the time when de Cugnac published his work, the wild grasses of the second group were assigned to various tribes typical of temperate regions; it is of particular interest to note that, more recently, the majority of these species have been reclassified, mainly from genetical considerations, and are now assigned to groups more nearly related to one or other of the typically tropical groups. Here, clearly, is an occasion where biochemical data not only amplify purely morphological observations, but even indicate a more natural grouping than that at first proposed by the more orthodox taxonomic methods.

Again, Colin & Belval[6] have investigated the common cereal grains with respect to their contents of raffinose and fructosans, and have established a tentative association between raffinose and fructosans in the germ. Thus rye, with a high content of fructosans, was found to contain a greater quantity of raffinose than did any other cereal investigated, whereas in oats both raffinose and fructosans had almost completely disappeared from the embryo by the time the grain had reached maturity. Wheat and barley were intermediate in their contents of these materials, and maize and sorghum, which contained no fructosan at any time, were also believed to contain no raffinose. Further, it was noted that raw cereals containing fructosans were characterized by high contents of β-amylase, whereas those lacking in fructosans showed virtually no amylolytic activity in the ungerminated grain. In confirmation of the facts of β-amylase distribution, Kneen[13] has found relatively large amounts of this enzyme in ungerminated rye, wheat and barley, with smaller amounts in raw oats; maize, sorghum and rice contained substantially no β-amylase.

Such findings are typical of those which may be derived from a comprehensive study of cereals but cannot be detected in an individual species; the suggested correlation between raffinose and fructosan content is of particular interest in that the amounts of both sugars present at any time may be the direct result of some third factor, probably enzymatic in nature, and certainly worthy of investigation, especially in view of the fact that a somewhat comparable pattern of distribution appears to exist in the case of the apparently unrelated β-amylase.

From a more practical point of view, it must be remembered that all the common cereals, or preparations from them, have at one time or another been used as mash-tun adjuncts, and although many of these may equally well supply starch for mash-tun conversion, or may function more or less satisfactorily as nitrogen diluents, all will not necessarily make an equal contribution of minor carbohydrate material to the wort; this fact in relation to the potentialities of various adjuncts as sources of viscous, gum-like material has already been discussed in some detail elsewhere.[27] The amounts of each individual minor component contributed may be small, but few would now deny the potential significance of trace quantities of certain materials. The purpose of the present study was therefore threefold: (*a*) to carry out a broadly-based survey of the sugars and oligosaccharides of cereal grains with a

TABLE I

FREE SUGARS AND OLIGOSACCHARIDES OF DIFFERENT CEREAL GRAINS

(Results in mg. of sugar or oligosaccharide per 100 g. of grain)

Sugar or Oligosaccharide	Barley	Wheat	Rye	Oats	Maize
Fructosans* ..	780	1,030	3,940	89	0
Raffinose.. ..	450	331	419	192	186
Glucodifructose ..	250	406	750	38	0
Maltose	90	†	†	†	†
Sucrose	908	836	1,857	639	783
Glucose	107	92	77	52	50
Fructose	26	57	98	91	55
Total	2,611	2,752	7,141	1,101	1,074

* Soluble in 80% ethanol. † Trace, insufficient for estimation.

view to furthering the investigation of metabolic changes in germinating barley; (*b*) to provide more detailed chemical analyses of cereal grains, and so to amplify present knowledge of the relationship between chemical composition and classification in the Gramineae; (*c*) by identification of minor carbohydrates of cereals and cereal adjuncts to indicate possible effects of these adjuncts on subsequent wort properties.

EXPERIMENTAL

Intact grains.—In general, analyses of the sugars and oligosaccharides of the five cereals, barley, wheat, rye, oats and maize were carried out in the manner previously described for barley.[15] With oats and maize, however, the high proportions of fat present in the extract proved troublesome at an early stage of concentration and a preliminary defatting of the ground grain in a Soxhlet apparatus was essential. The trace-quantities of sugar found in the petroleum-ether extract were readily removed by shaking with water, and these washings were added at a later stage to the main bulk of aqueous extract. After total reducing sugars had been estimated on suitable aliquots of the extracts, preliminary chromatograms were prepared and sprayed with aniline oxalate to detect all sugars (but especially aldoses), with α-naphthol in phosphoric acid to detect ketoses, and with ammoniacal silver nitrate to detect reducing sugars. Results of this preliminary work with aniline oxalate spray are given in Fig. 1, whilst quantitative estimations of the individual sugars are given in Table I.

The only surprising feature of the results was the appearance, on chromatograms prepared from maize extracts, of a spot in the typical raffinose position. The material in this spot reacted with the various spraying reagents in the manner characteristic of raffinose, but in view of the statement of Colin & Belval[6] that raffinose is absent from maize embryos, a more detailed investigation seemed desirable. Material eluted from the raffinose region of the chromatogram was therefore subjected to acid hydrolysis and to invertase action. When the products of acid hydrolysis were separated in phenol-water and in butanol-acetic acid-water, monosaccharides were detected exactly opposite the reference spots of glucose, fructose and galactose; after invertase action a spot appeared opposite known fructose, and a spot presumably representing a reducing disaccharide composed of glucose and galactose was observed in the melibiose region. These observations may not unequivocally identify raffinose, but it may be definitely stated that, in this sample of maize, there was present a non-reducing trisaccharide containing glucose, fructose and galactose residues and, presumably, with the fructose β-furanosidically linked at the end of the chain; in view of the undoubted presence of raffinose in other cereals, it seems highly probable that this trisaccharide is present in at least some samples of maize.

In maize preparations also, a spot in the glucodifructose region was observed to give a definite reaction with aniline oxalate and with ammoniacal silver nitrate, but no reaction with α-naphthol; this behaviour,

which excluded glucodifructose, pointed to the presence of a disaccharide (melibiose?) resembling that formed by the action of invertase on the maize trisaccharide. The presence of this material and, additionally, of small quantities of galactose, suggested that some degree of autolysis had occurred during post-harvest storage. Examination of a second sample of maize showed that the occurrence of this disaccharide and of galactose was not a regular feature, as, on the chromatograms prepared from the second sample, glucose, fructose, sucrose and presumed raffinose were the only sugars detected.

None of the cereals investigated (Table I) contained more than approximately 0·2% of free reducing sugars, and although the presence of traces of maltose was obvious in all, only barley contained quantities sufficient for accurate estimation.

Glucodifructose and the other fructosans are particularly interesting in their distribution. Completely absent from maize, these oligosaccharides account for less than 15% of the total "sugar" content of oats, but for *ca.* 50% of that of wheat and barley and for almost 70% of that of rye. It may be noted, too, that the sample of Scotch oats analysed contained a high proportion of green-tipped, unripe corns. The fructosan content of oats is said to diminish in the grain as ripening proceeds,[6] and it is probable that dead-ripe material would contain quantities of fructosans even lower than those reported here. To sum up, it may be said that maize is exceptional among the cereals examined in containing no trace of fructosans whatsoever; oats is remarkable for the presence of trace quantities of fructosans, but wheat, barley and rye show, in varying proportions among themselves, similar ranges of sugars and oligosaccharides, comprising fructose, glucose, sucrose, maltose, glucodifructose, raffinose and fructosans.

Cereal adjuncts.—Commercial samples of barley flakes, maize flakes, oat flakes and rice grits were available for analysis, and a qualitative study of the sugars present in these adjuncts was carried out. Microscopic examination revealed that the rice grits were purely endospermic in origin, whereas the flaked products all contained varying proportions of extra-endospermic material. Thus, in maize, although no germ could be detected, remains of the pericarp-testa were obvious; in barley and oats the entire grain—husk, germ and endosperm—was represented in the flaked material. It was to be expected, therefore, that the oat and barley flakes would contain the sugars and oligosaccharides present in the whole grain, together with degradation products formed as a result of the pre-cooking process which is usually carried out prior to flaking. With maize and rice, on the other hand, sugars and oligosaccharides initially present in the endosperm, together with starch degradation products, would appear to be the only possible contributors to the free-sugar fractions of the respective adjuncts. Results of the qualitative analysis of the adjuncts are shown in Table II and in Fig. 2. All contained free raffinose, sucrose, glucose and fructose. Fructosans were present in barley flakes and in oat flakes, but were absent from maize flakes and from rice grits. Maize flakes yielded four spots of fructose-free material of lesser mobility than sucrose and these presumably represented degradation products of starchy or dextrinous origin; any such material, if present on the chromatograms from oat flakes or barley flakes, would have been obscured by the fructosans. Maize flakes also differed from the other cereal adjuncts in containing free arabinose and a pentose-containing oligosaccharide of approximately the same chromatographic mobility as maltose.

Discussion

Before attempting to assess the implications of the results presented above, it may be useful to refer to a number of other studies of carbohydrates and of enzymes concerned in carbohydrate metabolism in the various cereals.

Carbohydrates.—As far as the sugars themselves are concerned, it is clear that older analyses of fructosan-containing grains will tend to show rather high figures for sucrose as a proportion of glucodifructose and other low-molecular fructosans would normally have been returned as sucrose. Apart from this inevitable discrepancy, the results quoted in the present investigation are of the same order as many of those previously reported (*cf.* Winton[30]). In maize, from which fructosans are absent, Cameron[5] has shown that there is a wide possible range of concentrations of sucrose and reducing sugars, the actual quantities present being governed mainly by genetic factors. The sample here

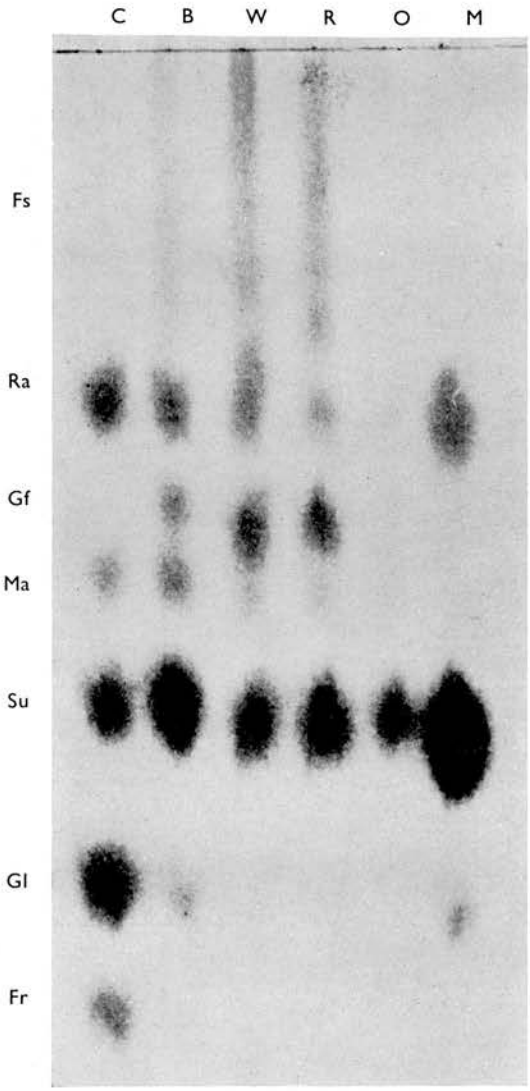

Fig. 1.—Sugars of the common cereals as shown by a chromatogram sprayed with aniline oxalate. C, control; B, barley; W, wheat; R, rye; O, oats; M, maize. Fs, fructosans; Ra, raffinose; Gf, glucodifructose; Ma, maltose; Su, sucrose; Gl, glucose; Fr, fructose.

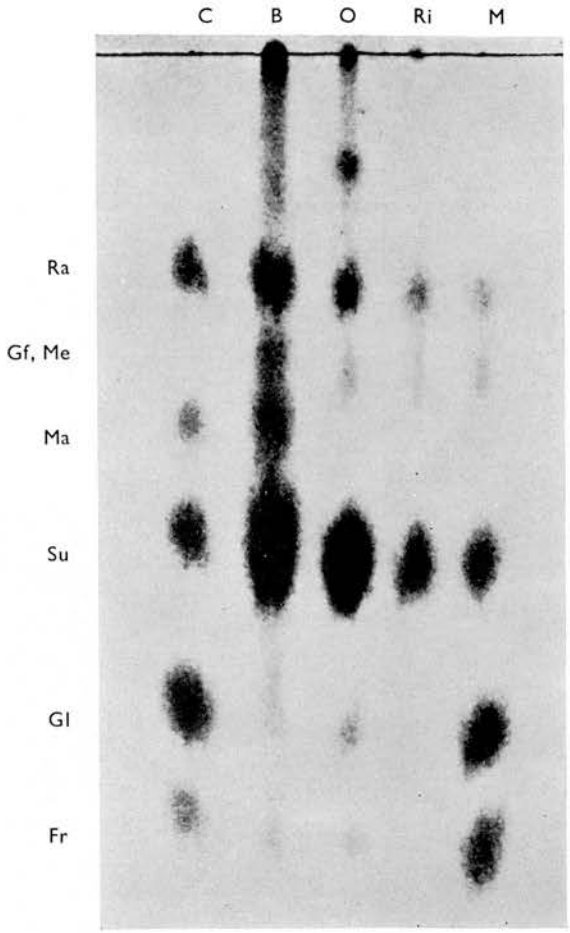

Fig. 2.—Sugars of cereal adjuncts as shown by a chromatogram sprayed with aniline oxalate. C, control; B, barley flakes; O, oat flakes; Ri, rice grits; M, maize flakes. Ra, raffinose; Gf, glucodifructose; Me, melibiose; Ma, maltose; Su, sucrose; Gl, glucose; Fr, fructose. For material above raffinose, see text.

TABLE II

FREE SUGARS AND OLIGOSACCHARIDES OF CEREAL ADJUNCTS

Reference position on chromatogram	Barley flakes			Oat flakes			Maize flakes			Rice grits		
	Reaction with spray*											
	1	2	3	1	2	3	1	2	3	1	2	3
Dextrins and Fructosans†	+	+	−	+	+	−	?	−	−	−	−	−
Raffinose	+	+	−	+	+	−	+	+	−	+	+	−
Melibiose and Glucodifructose	+	+	−	+	+	−	+	−	?	−	−	−
Maltose	+	−	+	?	−	−	?	−	−	−	−	−
Sucrose	+	+	−	+	+	−	+	+	−	+	+	−
Glucose	+	−	+	+	−	+	+	−	+	?	−	?
Fructose	+	+	+	+	+	+	+	+	+	−	−	−
Present	Fructosans Raffinose Glucodifructose Maltose Sucrose Glucose Fructose			Fructosans Raffinose Glucodifructose Maltose (?) Sucrose Glucose Fructose			Dextrins (?) Raffinose Melibiose (?) Maltose (?) Sucrose Glucose Fructose			— Raffinose — — Sucrose Glucose (?) —		

*1 = aniline oxalate
2 = α-naphthol in phosphoric acid
3 = ammoniacal silver nitrate

†+ = positive reaction
− = negative reaction
? = doubtful

investigated would appear to be a rather typical one of dent corn.

Of other carbohydrate constituents, little can be said about starch although it accounts for 60% or more of the dry weight of each cereal. Morphologically, as has already been noted, the starch grains of barley, wheat and rye are very similar; in maize, the close packing in the horny layer of the endosperm causes the individual grains, which are rather smaller than those of barley, to assume polygonal forms, whilst the less-tightly packed grains in the floury endosperm are more rounded. In rice and oats the starch grains are small and grouped into aggregates.[30] Information is available concerning the chemical nature of some of these starches. It is not clear what significance attaches to differences reported for amylose content; both wheat and barley starch have been stated to contain 19% of amylose, but the figures for maize and rice have been given as 23 and 15% respectively[4,18]. Similarly, the numbers of glucose residues per non-reducing end-group may show variation; the figure for barley may be *ca.* 26, though for the other three it is 20–21. Little, perhaps, except close similarity should be assumed from these figures.

The water-soluble hexosan and pentosan contents of the cereals, however, are by no means similar in the different species. Water-soluble pentosans were present in all the cereal grains examined by Preece & Hobkirk,[25] but whilst rye contained 350 mg. of water-soluble pentosan per 100 g., oats contained only one-seventh and maize only one-tenth of this amount, with wheat and barley intermediate. Some degrees of association is apparent between the contents of water-soluble pentosan gum and of fructosan, with rye, wheat, barley, oats and maize representing the order of decreasing concentration of both classes of carbohydrate material.

The fructosans of cereals in general range in size from a lower level of three hexose units to an upper limit of *ca.* 20 units, and no water-insoluble fructosan has been reported from the Gramineae. The pentosan gums, on the other hand, represent materials of a considerably greater degree of complexity; they are highly viscous in solution, and probably have molecular weights of *ca.* 30,000–40,000,[24] representing a degree of polymerization of 200–300 pentose units. Furthermore, there is every reason to assume a close structural relationship between these water-soluble

materials and the typically insoluble hemicelluloses. Again, fructosans are essentially storage carbohydrates which may be degraded and metabolized, whereas the pentosans are generally considered to stand in some relation to the structural part of the plant, *viz.*, the cell walls. To suggest a possible connection between a reserve food and apparently inert material associated with the cell wall may at first sight appear to be rather far-fetched, but it must be remembered that both the cell-wall materials and the carbohydrate reserves are formed as a result of protoplasmic activity; the fact that rye, for example, contains a high concentration of fructosan and pentosan materials intermediate in complexity between the monosaccharides and the high-molecular insoluble polysaccharides, whereas maize contains only trace quantities of these water-soluble materials, must surely be a reflection of the very different enzymic equilibria obtaining in these very different cereals during the ripening of the grain.

Apart from this possible association between pentosans and fructosans, there are some other points worthy of notice. The water-soluble β-glucosan characteristic of barley grains is found only in trace quantities if at all in the closely-related cereals, wheat and rye, but similar material is present in noteworthy amount in oats though not in maize. With the exception of barley, where soluble galactan content is extremely low (6 mg. per 100 g.) and raffinose content high, the amounts of raffinose show some slight degree of association, low galactan content and low raffinose content tending to go together.

Enzymes.—The enzymes which have been subjected to the most detailed study in cereal grains are the amylases. Kneen[13] has shown that whereas barley, wheat and rye possess high levels of β-amylase activity, oats show a very distinctly lower level, and it is possible only with difficulty to detect β-amylase in ungerminated maize and rice. α-Amylase activity, on the other hand, is of the same order of magnitude in all these cereals, only trace quantities being detectable in the raw grains, with the major development of this enzyme taking place after some 3 or 4 days of germination. Maltase seems to be present in the majority of cereal grains,[30] though no information is available as to the relative activity in the different species, and invertase appears to have been studied in detail only in barley, where a real, though low level of activity can be detected in the mature grains.[1] Information on the cytolytic enzymes, though of limited quantity, is of interest. Preece & Aitken[23] have shown that ungerminated barley contains a "cellulase" capable of degrading pure β-glucosan, but, unfortunately, as yet no study of the possible presence of such an enzyme in the other cereal grains has been made. Again, A. J. Brown[3] indicates that ungerminated oats contain an active "cytase" capable of degrading cell walls, though no such enzyme can be detected in ungerminated barley; cytase activity similar to that of raw oats is readily observed in "green" barley malt.[24]

Of the enzymes known to be of particular importance in synthesis, only phosphorylase has been studied in detail in any of the cereal grains (Porter[20]). This enzyme, which has been shown to be inhibited by β-amylase, is present in ungerminated barley; it would be of interest to determine whether phosphorylase can be more easily detected in oats, where β-amylase activity is very much less than in barley, or in maize from which β-amylase is substantially absent. Finally, though they do not stand in any direct relationship to the carbohydrates, certain of the oxidative enzymes of cereals are of interest in their distribution. Wallerstein, Hale & Alba[28] have studied *o*-phenylenediamine oxidase and pyrogallol oxidase and peroxidase; the first two of these enzymes were present in noteworthy amounts in wheat and barley, but appeared to be absent from maize and rice; peroxidase activity also was high in wheat and barley but low in maize and rice.

From the above brief and lamentably incomplete account, it can be seen that here again rye, wheat and barley form a natural trio, containing relatively high proportions of those enzymes which have been comprehensively investigated, *viz.*, β-amylase and certain oxidases. Maize and rice differ markedly from the three members of the Hordeae, and oats, though apparently little studied from the point of view of enzymes concerned in carbohydrate metabolism, stands in an intermediate position. In passing, it may be noted that both oats and maize contain a high proportion of oil in the ripe grains; it is possible that the diminishing free sugar content in ripening oats may be to some extent at least associated with the

activities of the lipogenic system of the grain.

General considerations.—It is clear that rye, wheat and barley resemble one another in minor carbohydrate constituents, in starch grain morphology, and in β-amylase content more closely than they do the botanically less-closely related cereal grains. This is not surprising: indeed, if taxonomically-related species did not show some degree of biochemical affinity it might reasonably be felt that either the methods of classification or the results of the chemical analyses were not above suspicion. Chemically speaking, the principal aberrant feature now discerned is the presence in barley of substantial quantities of β-glucosan and the virtual absence of this gum-like material from rye and wheat. The fact that an apparently similar β-glucosan is also a feature of oats, would, no doubt, be explained by taxonomists as a case of parallel evolution, but little can be said on this topic until a fuller chemical study of the glucosans from both sources has been made.

However, in view of the close relationship obtaining among different cereals, it should be pointed out that it is desirable that any theory attempting to explain the carbohydrate characters of one should, in general terms at least, be applicable to the other two. Thus, the structure proposed by Perlin[19] for araboxylan of wheat is consistent with all the facts observed for the behaviour of soluble pentosan gum fractions from wheat; it fails when applied directly to pentosan fractions similarly obtained from rye and barley. Any proposed structure must, on present evidence, be merely tentative, but, for a working hypothesis, it would be desirable to adopt a concept capable of application to the pentosans from all three cereals, as is attempted by Preece & Hobkirk.[25] The proposed modification of the Perlin hypothesis is slight, but by assuming fundamental xylan chains of different length (and, therefore, of different inherent solubility quite apart from the degree of association with araban), the observed results for all the cereals studied seem capable of rationalization, whilst permitting recognition of a similar type of molecular organization from one cereal to another.

Whilst recognizing that such dangers of interpretation may exist, it still seems legitimate—at least in the first instance—to use rye, wheat or barley (whichever is most convenient) for preparation of any of the minor carbohydrate constituents of these cereals. Thus, solubility relationships have so far precluded the preparation of purely pentosan gum from barley or wheat, though such "pure" pentosan can be obtained from some (though, perhaps significantly, not from all) samples of rye; study of pentosan relationships may accordingly be facilitated by using the rye product. Again, to investigate soluble galactan from barley where only 6 mg. per 100 g. is present would be most arduous; rye, containing ten times as much, should again be a more suitable starting material. The value of such "analogous" investigations may be very great, so long as the need for rigorous cross-checking of results from one cereal to another is kept in mind when drawing final conclusions.

Finally, it is worth while to consider what is the exact biochemical status of a particular cereal at the point when it is judged to be ripe. Studies of the carbohydrates of ripe grains are of necessity incomplete, and detailed information derived from analyses carried out during ripening, to maturity and throughout germination might be expected to give a clearer picture of the metabolic significance of the sugars, oligosaccharides and gums. Thus, fructosans become depleted in amount as cereals ripen and free sugars are utilized in respiration; the amounts of different carbohydrates in ripe grains may largely represent the residues from preharvest metabolism, though affected also by post-harvest treatment. The picture which ultimately emerges may well show entirely similar fundamental mechanisms in all cereals, but with shifts in the metabolic emphasis in different individuals. Do fructosans persist in rye and barley because "ripening" has not taken these cereals so far in a particular direction as it takes oats? Is water-soluble β-glucosan, which is almost completely eliminated during the early stages of barley germination,[26] substantially absent from ripe rye and wheat because it has already been converted into something else? That variations in the status of "ripeness" may account for conflicting reports concerning the existence of α-amylase in raw barley has been suggested elsewhere (Preece[21]), whilst it has been noted that not all samples of rye are equally suitable raw materials for the preparation of pentosan gum free from hexosan. These considerations would appear to be of very great significance, and to be

able to answer the points raised would represent an enormous advance in knowledge of cereal metabolism.

To progress any further in understanding the carbohydrate metabolism of barley, therefore, the most immediate requirement would seem to be a fuller knowledge of the various enzyme systems operating both during grain ripening and during the early stages of germination. Further studies in development of the present series are therefore being devoted principally to the enzymatic relationships involved, rather than specifically to the free sugars. Apart from post-harvest changes which would appear to be minimized by combine harvesting, these sugars, in common with other grain constituents, merely represent by their amounts the balanced effects of various enzyme systems prior to, and at, a particular point of time,—the point at which the grain in question is arbitrarily adjudged to be ripe.

Cereal adjuncts.—The nature of the sugars and oligosaccharides present in the various cereal adjuncts proved to be very much what might have been deduced from a knowledge of the composition of the entire grains. It is probable that the majority of these substances are of no great significance in the brewing process, except as additional sources of fermentable carbohydrate and, possibly, of unfermentable dextrins. To this general statement, however, there is one important exception—raffinose. This trisaccharide is absent from malt when this represents a product from substantially fully-viable barley,[17] and it is not found in the worts from such a malt[10,11]. Addition of cereal adjuncts to the mash, however, necessarily involves addition of a small, but definite amount of raffinose. Although any invertase present in the mash would catalyse at least partial hydrolysis to fructose and melibiose, the latter sugar must be expected to pass forward into the fermenting wort and, in the case of a normal top-fermentation, into the finished beer.

Raffinose is present mainly in the embryo of cereal grain[6,7] and it must be presumed that cereals where the whole grain is used for flaking will make the greatest contribution of this trisaccharide. No quantitative estimation of raffinose in different adjuncts has been made, but from a knowledge of the intact grains, and of the disposition of the sugars therein, it may be suggested that barley flakes are outstandingly rich in this sugar and will therefore pass on a notable amount of melibiose to the wort. It will be recalled that Wiles[29] has recently described a number of haze-producing wild yeasts (strains of *Sacch. carlsbergensis*) which differ from British top-fermentation yeasts in their ability to ferment raffinose and melibiose completely. It is possible that use of flaked barley as a mash-tun adjunct might permit the growth of these wild yeasts, by virtue of the melibiose or unchanged raffinose persisting in the finished beer. The amount of raffinose is certainly not great; the mean value for 10 samples of barley[15] was 450 mg. per 100 g., and, assuming the use of 15% barley flakes in the grist, 100 ml. of a finished beer of O.G. 1040 might be expected to contain 5–10 mg. of melibiose. Whether this is a sufficiently high concentration to be of any significance for the development of haze-forming yeasts cannot be stated precisely, but it would be of interest to know whether there is any correlation between the occurrence of such yeasts in the beer and the use of unmalted cereals, and particularly barley, in the mash.

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IX

Barley Carbohydrate Metabolism in Relation to Malting

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A grain of barley is an extremely complicated structure, both botanically and chemically, and very many interacting factors, not all of which have yet been clearly formulated, are involved in the apparently simple procedure of turning barley into malt. Arbitrary restriction of attention to one facet of barley composition and behavior may be an essential preliminary to an integrated understanding of the whole grain, but the inevitable narrowness of a specialist's approach can be dangerously misleading, unless one continually remembers that every component of a biological system such as a germinating seed may interact chemically, metabolically and even structurally with every other component. The carbohydrates, however, constitute a group of allied substances of peculiar importance in malting: they are almost the exclusive source of fermentable extract, they provide the principal respiratory substrates and ultimate stores of energy for enzyme synthesis, and, on the debit side, some of them may act as impediments to rapid and complete modification.

In recent years increasing attention has been paid to the carbohydrates of barley, and the time now seems opportune to survey different members of the group and to offer an appraisal of their importance in relation to malting. Such a broadly based examination of chemical and biological problems of carbohydrate metabolism cannot provide great depth of detail. On the other hand, such a very general survey can often best reveal what the relevant problems are, and can indicate which ones have been more or less solved and which are still in need of further exploration.

The Barley Kernel

Carbohydrates account for over 80% of the dry weight of barley, but not one of the different component sugar and polysaccharide fractions is distributed randomly throughout the various tissues. Consider first the very diverse origins of the different regions of a barley grain. The husk, or hull, investing the kernel is derived from glumes — modified leaves which originally surrounded the flower — which persist to envelop the enlarged fruit at maturity. The husk is thus the oldest part of the grain. Though it is photosynthetically active during ear development, by the time the grain is ripe the tissues of the husk are dead and their cells are virtually depleted of protoplasm. Enclosed by the maturing and then dying husk, the ovary enlarges rapidly: the fertilized ovum divides and the newly formed cells, densely packed with cytoplasm and each equipped with a large and potentially active nucleus, differentiate to form the rootlets, the shoot, and the scutellum. This embryo, highly organized physiologically as it is, accounts for only 3% or thereabouts of the dry matter of the grain. Concurrently with the elaboration of the embryo there is a burst of creative activity, instigated by the triple fusion nucleus, which results in the formation of a tissue differing very markedly from the meristematic embryo. New nuclei form in rapid succession; these lie at first in a fluid matrix containing young starch granules. Subsequently, and in rather a haphazard fashion, partitions are laid down which subdivide the growing endosperm into cellular units. The final result of this activity is an essentially starchy endosperm bounded on all sides, except the proximal one abutting on the scutellum, by two or three layers of thick-walled cells containing protein and fat but devoid of starch. Growth of the endosperm may be exuberant, but it is limited in extent. Eventually the capacity for nuclear division is exhausted and respiration sinks to a negligible level; nuclei and cytoplasmic particles can still be detected among the starch granules of the ripe grain but they are apparently metabolically inert. The grand period of endosperm activity, however, suffices to form over 80% of the grain.

A kernel of barley thus stands at the crossroads of two generations: it contains tissues surviving from its parent in the form of the senescent husk and traces of the investing integuments and original matrix of the ovule; cells which are still wholly juvenile and which constitute the embryo; and endosperm cells with degenerating triploid nuclei hidden among closely packed starch granules. The integumentary layers have played their part in grain formation, and in their old age they harbor an extensive range of catabolic curiosities — anthocyanogens and suberin and the like — which exert complex effects on such widely diverse matters as oxygen intake by the germinating grain and haze formation and bitterness in the finished beer. The cells of the rootlets and shoot of the embryo resemble other meristematic cells (about which, incidentally, a great deal remains to be learned), whereas the scutellum, though apparently cytologically similar to the remainder of the embryo, has minimal potentialities for cell division and maximal capacities for elaboration and secretion of hydrolytic enzymes. The third major constituent of the grain, the endosperm, is

an innovation peculiar to flowering plants. It seems to act as a sort of nursemaid to the embryo, supplying it with food, and, in certain plants, with growth-promoting factors. In barley, selection of high-yielding varieties throughout the ages has profoundly altered the character of the endosperm, so that it contributes some 65% of starch to the grain, compared with the modest 12% contribution in certain wild grasses. Some idea of the anatomical heterogeneity of a barley kernel is given in Figure 1.

General carbohydrate analysis

Not surprisingly, the carbohydrate composition of these different regions of the grain is at least as varied as their histological structure; hence any gross analysis of barley, essential though it is as a starting point for further investigations, is not of itself very revealing. The values quoted in Table I do, however, indicate the total amounts of certain different sugars and polysaccharides which have been extracted from whole barley and characterized satisfactorily. Broadly speaking, these carbohydrates can be subdivided into sugars and oligosaccharides, starch, hemicelluloses, and crude cellulose. Methods developed for their separation and identification are also indicated in the Table. The figures cited must all be regarded as approximations, for they have been abstracted from different analyses performed by several different investigators. However, the total of all the individual components listed probably accounts for nearly three-quarters of the dry matter of the grain, and there remains an elusive 5% or so of carbohydrate material which is not accounted for. This falls into an analytically awkward category of substances which avoid extraction by reasonably gentle solvent action, but fail to survive the drastic operations involved in determining crude cellulose. That this material is almost certainly pentosan is clear from the discrepancy between the pentosans variously accounted for in the extracted or residual carbohydrates of Table I (2.2%) and the value of approximately 8% of pentosan derived from furfural determinations carried out with unfractionated grain (1).

It will be noticed that one common group of plant polysaccharides has been completely omitted from the general analytical scheme — the pectic materials. Values have indeed been quoted for barley pectin (2) ranging from 1% down to 0.005%, but the difficulties involved in this particular analysis are so great that the status of barley pectin can presently at best be regarded as highly equivocal.

Characteristics of the carbohydrate fractions

Little need be said about most of the carbohydrates listed in Table I, but possibly a brief consideration of some of the less familiar components may be pertinent. The fructosans (3), for example, constitute an interesting homologous series based on sucrose and ranging in complexity from a trisaccharide, glucodifructose, to a polymer containing one glucose and ten or more fructose residues. Fructosans form an important reserve in the internodes of the stem and, as Harris and MacWilliam (4) have shown, they contribute largely to the labile reserves of the ear during the first three weeks of its development. With

TABLE I

SOME CARBOHYDRATE FRACTIONS OF WHOLE BARLEY

Fraction and Method of Preparation	Yield % Dry Weight	Method of Analysis or Examination	Individual Components % of Fraction	
Sugars and oligosaccharides extracted by boiling 80% ethanol (12)	3.6	Paper partition chromatography	Fructosans Raffinose Sucrose Hexoses	33 16 44 7
Water-soluble hemicelluloses extracted from sugar-free residue at 40 C. (9)	1.1	Fractionation by precipitation with ammonium sulfate	β-glucan Pentosan Traces of mannan and galactan	73 24
Starch extracted by perchloric acid (4)	66	Amylose determined as iodine-complex	Amylose Amylopectin	ca. 20 ca. 80
NaOH-soluble hemicelluloses extracted from starch-free residue by 4% NaOH at room temperature (13)	2.2	Partial fractionation by precipitation with ammonium sulfate	β-glucan Pentosan Uronide	36 53 1
Crude Cellulose residue from treatments with hypochlorite and sulfite (6)	4.5	Constituent sugars determined after solution in 72% H_2SO_4 and subsequent hydrolysis in N-acid	Glucan Xylan Mannan	70 10 20

References: MacLeod (12); Preece *et al.* (9); Harris *et al.* (4); Preece *et al.* (13); MacLeod *et al.* (6).

accelerated starch synthesis in the ear, however, the fructosans assume an increasingly subordinate position.

Barley starch cannot be considered an unfamiliar carbohydrate, for it has been intensively studied both chemically and enzymically, but it is worth noting in passing that the biophysical processes involved in elaborating the very familiar starch granule are still completely obscure. Equally obscure are the developmental changes involved in the transformation of a predominantly fructosan-storing vegetative barley plant to the starch-forming fruiting condition: possibly the triploid condition of the endosperm nuclei may be of importance in this change of metabolic emphasis. Recent work (5) has suggested that much of the reserve starch of the endosperm is the result of direct photosynthetic activity of the ears themselves rather than, as had formerly been assumed, the final polymerization of sugars translocated from the stem and leaves. The structure of the husk and its awn and even of the ovary wall, and the contribution of these tissues to grain filling, are thus possibly worthy of some consideration in relation to selection of new varieties for yielding potential.

The remaining carbohydrates of Table I which merit discussion are those loosely assigned to the plant cell walls: cellulose and pentosans are well-known wall components, though the biological mechanisms involved in their synthesis are still wrapped in mystery, and the term 'crude cellulose' can include a great many fibrous polysaccharides — in this case mannan and xylan (6) in addition to genuine cellulose, 1,4 β-linked glucan. The water-soluble hemicelluloses are generally discussed along with cell-wall materials, although their location, and particularly that of the β-glucan (a viscous polymer containing an approximately equal number of β 1,3- and β 1,4-linkages), has not been definitely established. These hemicelluloses or gums have been the subject of a series of investigations by Meredith in Winnipeg (7, 8) and by Preece in Edinburgh (9, 10, 11) and there is no doubt that their importance in malting is very much greater than their proportionate contribution to the grain carbohydrates would suggest.

Distribution of carbohydrates within the kernel

In addition to fractionating the carbohydrates of the whole grain, it is also possible to some extent to fractionate the grain itself and then to apply the types of analysis discussed above to the separate sub-fractions. The first major difficulty which arises in this type of study is a purely mechanical one which has not yet been satisfactorily solved and which arises from the anatomical structure of the grain. The vascular bundle linking the ovary with the rachis and, more especially, the transfusion or sheaf cells which radiate out from the vascular tissue, penetrate deep into the endosperm from the furrow of the grain and defy all efforts to remove the husk and pericarp-testa cleanly from the endosperm. These structural features are shown in Figure 1, and their occurrence doubtless explains the failure of analysts to develop a reliable method of estimating husk content by physical means. However, although all the husk cannot with certainty be separated from barley, reasonably pure samples of husk can be secured by the use of a restricted milling process. More extensive milling leaves pearl barley containing over 95% of starchy central endosperm. A third fraction, the embryos, can be isolated by dissection in sufficient numbers to permit at least a general analysis of carbohydrate constituents. Obviously, there are also unfortunate omissions in this scheme of grain fractionation. Thus, no satisfactory preparations of aleurone cells have yet been achieved, and the microscopic appearance of the cell walls of this layer (Figure 1) suggests that a carbohydrate analysis of a pure preparation of aleurone might be of considerable interest.

However, when series of carbohydrate analyses are performed on those regions of the grain which can be prepared as reasonably pure and homogeneous tissues, certain striking differences are at once apparent in the localization of different carbohydrate fractions. Table II gives a summary of the distribution of selected barley carbohydrates in the husk, the embryo, and the central endosperm. In spite of the unavoidable omissions both in fractionation and in analysis, a number of interesting points emerge immediately. For example, although hexose sugars are fairly evenly distributed through both embryo and endosperm, over 80% of the metabolically important oligosaccharides raffinose and sucrose is concentrated in the embryo (12). This latter region of a barley grain derives approximately one-fifth of its dry mass from primary reserves of free sugars — a phenomenally high sugar concentration for any plant organ. Cellulose, which is abundantly present in the husk and readily detected in the embryo, is virtually absent from the central endosperm cells (6). Admittedly, traces of cellulose can be recovered from pearl barley, but careful observation of this material during successive treatments with hypochlorite and sulphite strongly suggests that the final residue originates almost exclusively in the fragments of husk-like material occluded in the pearled grain.

The ethanol-soluble substances, *i.e.*, the sugars and oligosaccharides, and the completely resistant fibers, *i.e.*, crude cellulose, can readily be separated from the various grain fractions, and starch, of course, is restricted to the endosperm. What about the rest of the carbohydrate: the more or less readily solubilized pentosans and β-glucan? Difficulties arise with these presumed cell-wall materials, for the mechanical effect of pearling is to degrade some of the starch, and the β-glucan is almost lost amid the overwhelming excess of artificially-produced α-linked dextrin. With the

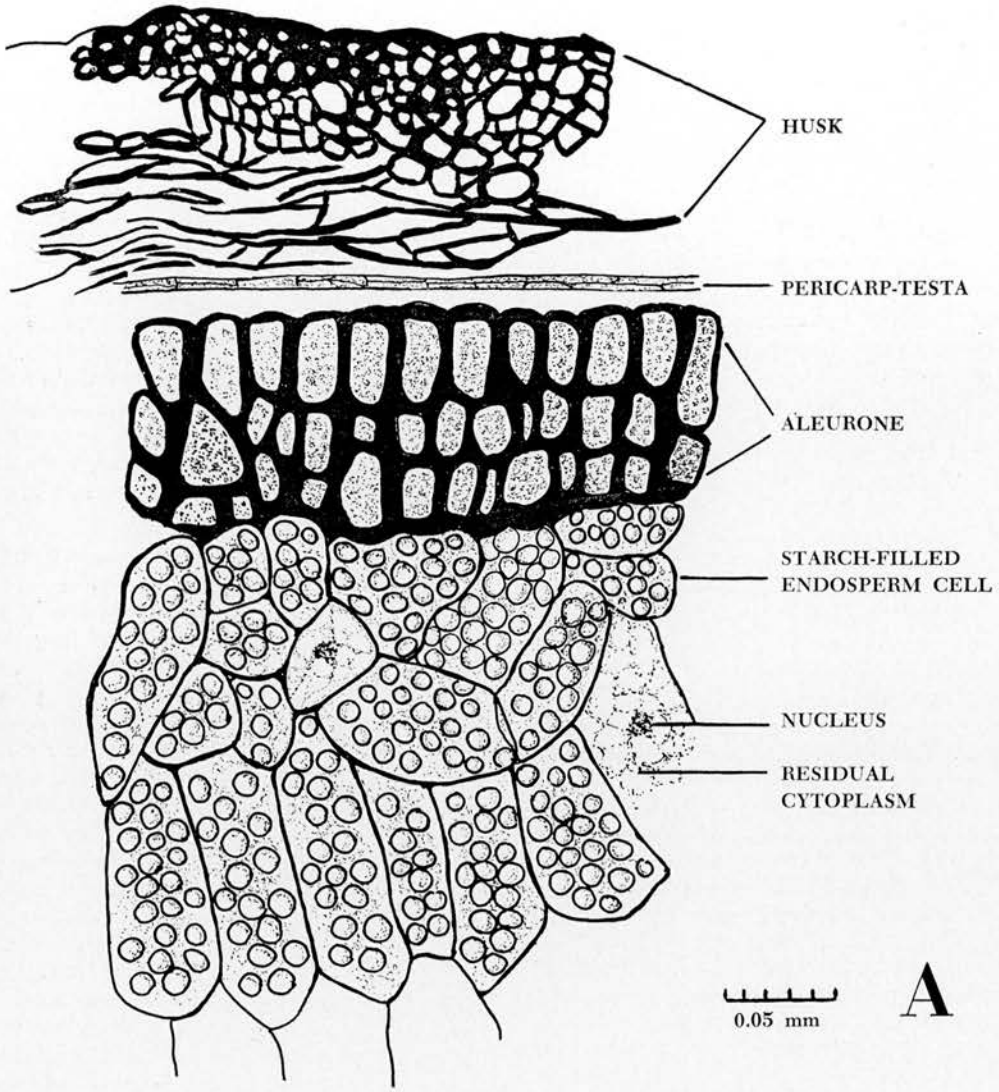


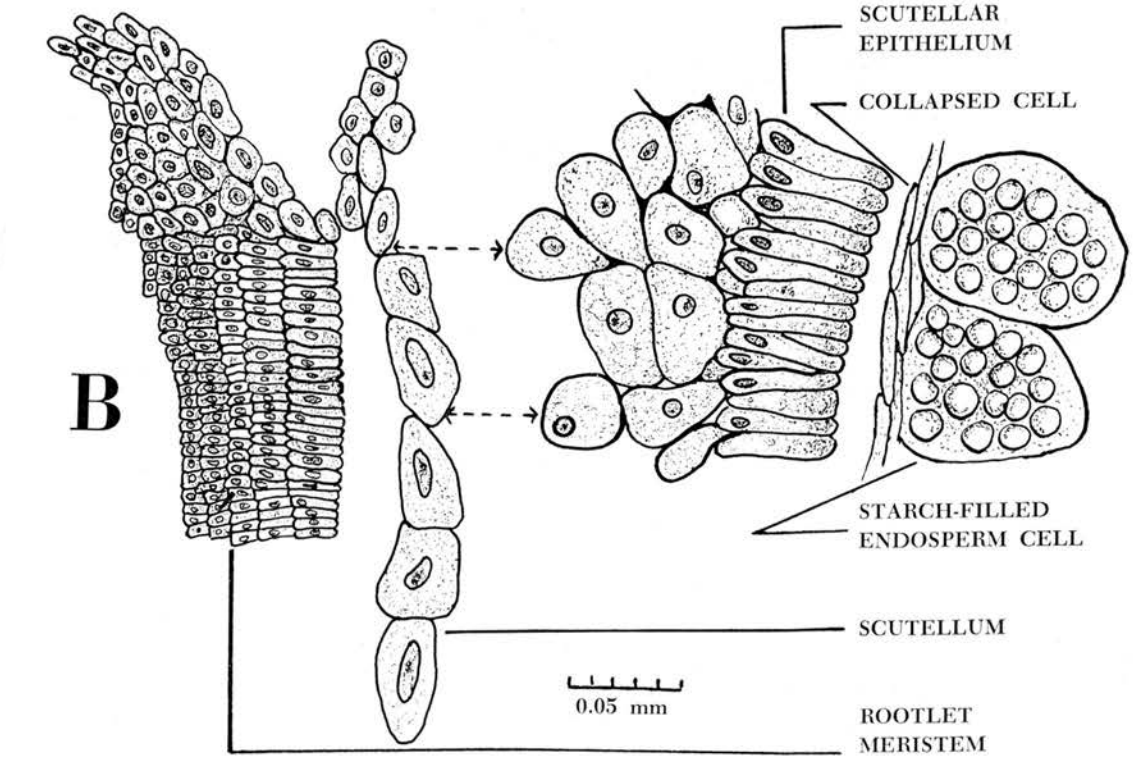
Figure 1

Various sections of a barley kernel

A — *transverse section of outer layers, showing cell walls of husk, aleurone and endosperm; residual nuclei and cytoplasm are shown in two of the endosperm cells.*

B — *Longitudinal section of embryo and adjacent endosperm, showing meristematic rootlet cells, absorptive scutellar epithelium and two endosperm cells.*

C — *diagrammatic representation of transverse section in the region of the furrow; the "sheaf" cells penetrate to the center of the endosperm.*


B
SCUTELLAR EPITHELIUM
COLLAPSED CELL
STARCH-FILLED ENDOSPERM CELL
SCUTELLUM
0.05 mm
ROOTLET MERISTEM

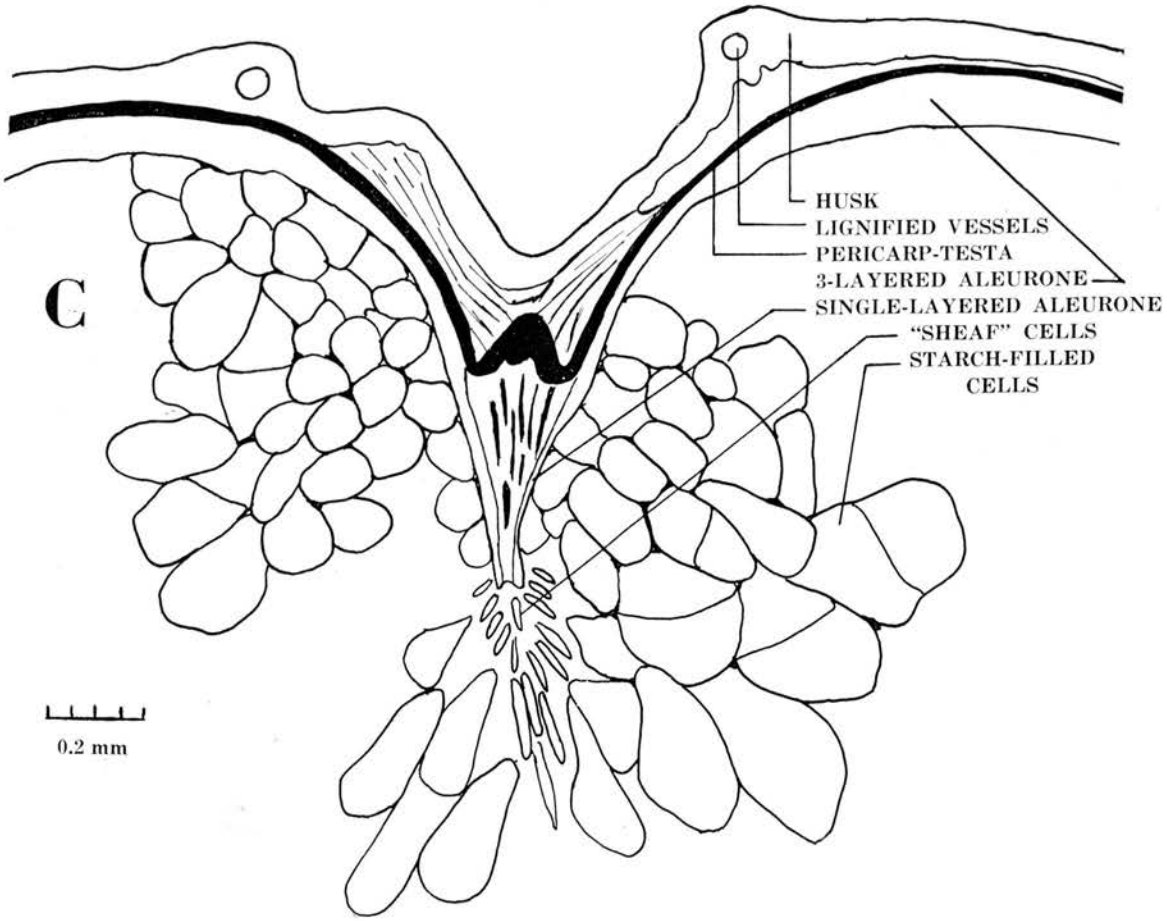

C
HUSK
LIGNIFIED VESSELS
PERICARP-TESTA
3-LAYERED ALEURONE
SINGLE-LAYERED ALEURONE
"SHEAF" CELLS
STARCH-FILLED CELLS
0.2 mm

TABLE II

DISTRIBUTION OF SOME CARBOHYDRATE FRACTIONS WITHIN THE KERNEL

Carbohydrate	Percentage Contribution to Dry Weight of Tissue		
	Husk	Embryo	Central Endosperm
Sucrose	0	14.0	0.20
Raffinose	0	10.0	0.08
Hexoses	0	0.2	0.26
Total β-glucan	Less than 0.02	0	1.7
Total pentosan	3.6	0.4	1.0
Galactan	0	0.3	0
Uronic acid	Present	Present	0
Crude cellulose	30%	7	0.4

0 = not detected.

The carbohydrate fractions of the second group are present in the grain in higher concentrations than those quoted in the Table: the figures given refer to extracted and partially purified materials.

pentosan, moreover, the failure of the fractionation scheme for polysaccharides to account for all of this component makes it impossible to draw any final conclusion about its disposition throughout the grain. However, it has been shown (13) that the alkali-soluble hemicelluloses of barley husk essentially resemble typical straw hemicelluloses, being rich in xylan and containing glucuronic acid; similarly-prepared material from starch-freed endosperm very largely resembles the soluble β-glucan and contains only a small amount of xylan.

Although analyses of whole grain for pectic materials have not given any very reliable figures, there can be little doubt that cereal embryos contain not only typical polygalacturonic acid but also the araban and galactan (14) so commonly associated with pectic acid. None of these materials could be detected in pearled barley (2), so apparently the central endosperm at least is virtually free of pectin and its usual concomitants. Comparisons between species can be misleading, but the ready demonstration that the individual cells of endosperms of certain wild grasses have no cohesive properties and therefore, presumably, no intercellular pectin, may be taken as supporting evidence for the absence of pectin from barley endosperms. It may also be observed that histochemical tests for pectin, though admittedly of doubtful validity, do give positive results for embryo and husk and negative results for the central endosperm.

To summarize, then, a grain of barley contains:

(*a*) a thick-walled partially lignified husk which is virtually free from water-soluble carbohydrates but liberally endowed with cellulose and hemicelluloses;

(*b*) a small, potentially active embryo, richly supplied with sucrose and raffinose, with cells enclosed in walls of cellulose and hemicellulose and separated by intercellular pectic material;

(*c*) a massive inert central endosperm which is relatively poor in sugars but rich in starch, with cell walls lacking cellulose but containing pentosans and β-glucan; and

(*d*) an outer endosperm layer, containing aleurone cells and sheaf cells radiating out from the chalaza, together with some starchy endosperm cells; this layer has not yet been analyzed.

Malting Changes

Sugars

Various investigations carried out to determine changes in free sugars during malting (15, 16), and partition of the sugars between endosperm and embryo all indicate that sucrose forms the principal carbohydrate respiratory substrate during the important early stages of growth initiation — though the possible importance of both fats and proteins as substrates should not be overlooked. Excised embryos can subsist for some time without extra-embryonic supplies of sucrose (14), mobilizing their own endogenous sources of oligosaccharides. As growth proceeds, the content of sucrose in the embryo is maintained by conversion of products of starch degradation translocated from the endosperm, a process which as Edelman *et al.* (17) have recently shown, involves the intermediary action of uridine diphosphate glucose in the scutellum. The formation of sucrose from glucose by barley scutella can proceed satisfactorily under anaerobic conditions — *i.e.*, in circumstances which might be expected to prevail within the moistened seed.

The fate of raffinose is less well understood. Raffinose utilization takes place only in the presence of oxygen (14), so that whereas in laboratory germination on filter paper all the raffinose is consumed within 24 hours following moistening of the grain, in malting, raffinose can usually still be detected at the completion of steeping. As soon as oxygen is available to the grain, however, raffinose utilization proceeds apace. The details of the aerobic metabolism of raffinose still await adequate explanation.

Little need be said about the metabolism of other sugars: fructosans can largely be hydrolyzed by invertase present in the scutellum (17) and thereafter, along with free hexoses, incorporated into the general synthetic and respiratory mechanism of the developing seedling.

β-Glucan

Fairly complete information is now available with regard to the fate of this viscous polysaccharide during malting, and detailed studies have been made of the rather complex group of enzymes involved in its degradation. Broadly, the picture is one of increasing solubilization of initially insoluble hemicellulose material, concurrently with enzymic cleavage of large molecules both by random scission and by stepwise attack from the end of the glucan chain, to release first mixed oligosaccharides and finally glucose. The development of the relevant enzyme systems has been intensively studied by Preece *et al.* (18), and it is clear that by the end of the malting process most of the viscous glucan has been successfully degraded so that the friable grain of malt is virtually free from this particular hemicellulose. It is difficult to escape the conclusion that β-glucan is associated with the endosperm walls and that its progressive removal is linked with their changing physical nature during modification; quite certainly no other polymer is so successfully eliminated during growth.

Pentosans

The fate of the pentosans, which have been more surely established as components of the endosperm walls, is rather less fully understood. Enzymes are present in barley which can release arabinose from the araboxylan molecules (11); others can catalyze breakdown of the xylan backbone in a fashion rather similar to that established for the breakdown of β-glucan. Levels of activity of the various pentosanases are low in ungerminated barley, and no spectacular increase comparable with the increase observed for some of the glucanases can be detected as malting proceeds. Enzyme preparations which degrade soluble araboxylan also show some slight but definite potentialities for attacking insoluble pentosans, but the actual degradation accomplished in malting must be minimal; comparative determinations (unpublished) of total insoluble pentosan in barley endosperm and in malt flour suggest a decline of only approximately 10%. To be sure, removal of a limited amount of structural material from a cell wall could easily allow its subsequent dissolution, and these changes in pentosan content, though quantitatively limited, may none the less be of the utmost importance.

Starch

Greenwood and Thomson (19) have recently published a comparative study of starches from a barley and its malt which shows a total loss of some 16% of the original barley starch during malting, together with a decline in granule size, a shortening of the external chains of the amylopectin and a very slight overall decline in the degree of polymerization of the amylose component. Enzymic attack on starch apparently is principally a β-amylolysis of external chains of amylopectin, which is believed to be concentrated on the outside of the granule; the change in amylose complexity is the result of a very limited α-amylolysis. The internal scission of amylose rather than of amylopectin may be due to the greater ease with which amylose is reached by solvents diffusing into the granule.

The question of solvent penetration has also been discussed by Preece (11) in relation to degradation of cell-wall components. It is encouraging to find that, now that the structure of the more important high polymers of barley is known and a reasonable body of information is available about the enzymes capable of catalyzing the degradation of these polymers *in vitro*, attention is being turned to the difficult problems of catabolism within the complex matrix of the cell.

Throughout the present general discussion of barley carbohydrates an attempt has been made first to analyze the relevant materials into chemically-defined entities and then to consider the grain itself as subdivided into reasonably homogeneous tissues, of different biological origins, to which different typical carbohydrates can with some certainty be allocated. Such an essentially analytical method of approach may serve as much to suggest problems as to solve them. Consider, for example, whether a synthesis of the results of the many meticulous studies of hemicelluloses and hemicellulases suffices to explain the changes which can actually be observed in the endosperm walls during malting. In theory, the activity of the extracted glucanases and even pentosanases appears to be more than sufficient to accomplish the changes observed microscopically. In practice, however, highly active carbohydrase preparations fail to solubilize the walls of isolated intact cells, although in the living grain these same walls are apparently dismantled with the utmost ease. The suspicion arises that some material other than carbohydrates is involved here, and the caveat expressed in the introductory paragraphs applies in this context in full force: understanding of specific, chemically-defined components, gained by precise *in vitro* experiments, is not sufficient to explain carbohydrate metabolism in the living kernel, where the interfering — or even possibly enhancing — activity of non-carbohydrate concomitants, which are rigorously excluded from exact biochemical determinations, may prove to be controlling factors. For a further mastery of carbohydrate behavior in malting, attention must now be concentrated on the non-carbohydrate components, with particular regard to their inter- and intracellular location.

Practical considerations

To end this discussion on a defeatist note would be misleading. A consideration of practical benefits which have accrued from what has been a more or less academic group of studies of important plant constituents is of interest.

As far as sugar metabolism is concerned, while it is intellectually satisfying to know the nature, disposition and rates of utilization of oligosaccharides of metabolic importance, this knowledge is likely to be of little direct use to the maltster. The presence of liberal amounts of sucrose in barley embryos may be associated with the smooth onset of seedling growth, as has been suggested (20), but it seems probable that the mechanisms available for utilization of fructosan and starch degradation products more than suffice for most malting barleys. However, if plant breeders use as parental material barley varieties which are outside the familiar malting types, then attention might have to be paid to this facet of carbohydrate composition, for there is a slight indication that the proportions of available sucrose may be genetically controlled.

With the appreciation that degradation of β-glucan is an integral aspect of the conversion of barley to malt, considerable attention has been paid to the potentialities of malting barleys for glucan solubilization. Sandegren (21) has postulated the desirability of a high level of "cytase" activity, as measured by degradation of a hydroxyethyl cellulose and Preece (22), thinking along similar lines, has investigated β-glucanase action in relation to available β-glucan substrate, and has concluded that a certain balance between enzyme and substrate in the barley is desirable. Although results of carefully controlled periods of autolysis gave some indication of potential malting behavior, this balance of enzyme and substrate seems likely to be important only in the earliest days on the floor, for glucanase synthesis during growth is so extensive that the substrate *per se* is unlikely to survive as an impediment to modification. However, the early days of steeping and growth are possibly the most important in malting, and here again barley breeders using unfamiliar varieties might find the autolytic estimation of the glucan-glucanase balance a useful exploratory technique.

The behavior of malt in the mash tun lies beyond the scope of this review, but it is interesting to note that Meredith (23) has been able to show some correlation between the cytolytic activity of green malt and a number of important properties of malt extract. Thus the influence of the soluble hemicelluloses and the enzymes associated with their degradation is not confined to malting alone, and, indeed, the possible influence of both β-glucan and pentosans on properties of beer foam is a matter of considerable interest.

Reverting now to barley carbohydrates, one of the most useful methods of relating carbohydrate composition of barley to extract of finished malt is the insoluble carbohydrate determination of Bishop & Marx (24). "Insoluble carbohydrate" is the residual material which survives precisely defined and strictly controlled treatment with dilute acid and dilute alkali. The amount of this residue, taken in conjunction with 1,000-kernel weight and nitrogen content, has allowed the formulation of an extract prediction equation of very general applicability. This empirical measurement estimates what Bishop has described as "carbohydrate complementary to extract" and includes cellulose, part of the hemicelluloses, and lignin; small amounts of fatty material, protein and ash are also determined in the insoluble carbohydrate. The particular merit of Bishop's equation lies in its applicability to unknown varieties, and its value to the plant breeder is thus obvious. Moreover, it makes allowance for the intractable pentosan material whose characterization has so far defied the techniques of the "pure" carbohydrate chemist, and it probably still represents the most useful single determination of an essentially carbohydrate component. One must remember, however, that it is an estimate based on statistical probabilities, and that occasional instances may be encountered when faulty conclusions may be drawn from the use of this one measurement. Bishop himself records an example. A hybrid appeared to contain pentosan-free polysaccharide which could be solubilized by the acid-alkali treatment, but which was immune to the germinating grain's own armament of enzymes, so that an excessively high prediction of extract was realized by using the standard equation. β-Glucan might here be implicated, and one wonders to what extent more modern methods of hemicellulose analysis, such as the autolytic technique of Preece (22), might have detected this anomalous behavior. It must be stressed, however, that such anomalies are apparently of rare occurrence.

The future

The most profitable lines for future attack on problems relating to barley carbohydrates and their metabolism are difficult to suggest. More exact knowledge of the endosperm pentosans, both those which are solubilized during malting and those which resist enzymic degradation, undoubtedly would be desirable. To know something about the structure of the walls of the aleurone layer cells and something about the mechanism of raffinose catabolism would be at least of academic interest, but a purely chemical and biochemical approach will, probably, not do much to further useful knowledge. Major advances can possibly best be expected from the application of histological techniques developed to aid in studying the non-carbohydrate concomitants of cell walls and of starch granules within the cells: paradoxically, then, the immediate future of research into barley carbohydrates may well depend on refinement of methods for recognizing non-carbohydrate components of the grain.

Summary

Barley carbohydrates constitute a group of substances of special importance in malting. They are the source of nearly all the fermentable extract, and they act as respiratory substrate and source of energy for enzyme synthesis; some of them may impede rapid and complete modification.

Among the carbohydrates of the barley kernel are sugars, oligosaccharides, starch, hemicelluloses, and crude cellulose; these account for approximately 75% of the dry matter of the kernel, with another 5% or so almost certainly consisting of pentosans. Fructosans are reserve material in the internodes of the stem; they are utilized extensively in the first few weeks of development of the ear. Barley starch is not an unfamiliar carbohydrate, yet the processes involved in elaboration of the starch granule and in transformation of the plant from the vegetative to the fruiting condition are still obscure. Recent work suggests that much of the endosperm starch is produced by photosynthesis directly in the ear. Elucidation of the relationship of the structure of husk, awn and ovary wall to grain filling may be of value in selecting new varieties for yielding potential. Although a relatively small proportion of the barley carbohydrates, the hemicelluloses and gums are of special significance in malting.

Barley husk, embryo and endosperm fractions show striking differences in carbohydrate composition. The embryo contains over 80% of the raffinose and sucrose. Cellulose, which is abundant in the husk and present in the embryo, is virtually absent from the central endosperm cells, whereas starch is found only in the endosperm. No definite conclusions can as yet be drawn concerning the distribution of pentosans and β-glucan among the parts of the kernel, owing to fractionation or analysis difficulties.

Changes in the carbohydrate constitution of the kernel during malting have profound effects on the properties of the finished malt. Among the free sugars, sucrose forms the principal respiratory substrate during the early stages of growth initiation. Raffinose is utilized rapidly if oxygen is available to the kernel. The hemicellulose β-glucan, which may be associated with endosperm cell walls, is progressively degraded during malting, the friable malt kernel being practically free of it. The fate of the pentosans is not yet well understood. Levels of the various pentosanases are low in ungerminated barley and do not increase considerably during malting. Although the extent of pentosan degradation must be concluded to be minimal, removal even of limited amounts of pentosan from the structural material of the cell walls could easily allow their subsequent dissolution. A loss of about 16% of starch in barley occurs during malting; starch granules decrease in size, the external chains of the amylopectin become shorter, and the degree of polymerization of the amylose declines slightly.

A useful method of relating barley carbohydrate composition to extract of finished malt is the "insoluble carbohydrate" analysis of Bishop and Marx, which estimates "carbohydrate complementary to extract." Use of this measurement and of the equation developed in conjunction with the method is of value in the evaluation of unfamiliar barley varieties.

In vitro experiments providing an understanding of the behavior of specific, chemically defined components do not suffice to explain carbohydrate metabolism of the living kernel, in which non-carbohydrate concomitants could well prove to be controlling factors. The immediate future of barley carbohydrate research may depend on refinements in the means of study of these non-carbohydrate concomitants.

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[*Résumé*]

Le Métabolisme hydrocarboné de l'Orge en Relation avec le Maltage.

Les hydrates de carbone de l'orge constituent un groupe de substances d'importance particulière au maltage. Ils sont la source d'à peu près tout l'extrait fermentescible, et ils agissent comme substrat respiratoire et source d'énergie pour la synthèse enzymatique; certains d'entre eux peuvent faire obstacle à une rapide et complète désagrégation.

Parmi les hydrates de carbone du grain d'orge il y a des sucres, des oligosaccharides, de l'amidon, des hemicelluloses, et de la cellulose brute; ceux-ci représentent approximativement 75% de la matière sèche de la graine, avec en plus 5% à peu près, consistant presque certainement en pentosanes. Les fructosanes sont de la matière de réserve dans les internodes de la tige; ils sont utilisés extensivement pendant les quelques premières semaines du développement de l'épi. L'amidon de l'orge n'est pas un hydrate de carbone peu familier, néanmoins, les processus impliqués dans l'élaboration du granule d'amidon et dans la transformation de la plante de l'état végétatif à l'état productif sont toujours obscurs. Des travaux récents suggèrent qu'une bonne partie de l'amidon de l'endosperme est produite par photosynthèse directement dans l'épi. L'élucidation de la relation existant entre la structure des téguments, des barbes, de la paroi ovarienne et le noyau du grain, peut être importante dans la sélection de nouvelles variétés du point de vue du potentiel de rendement. Bien que ne représentant qu'une proportion relativement petite des hydrates de carbone de l'orge, les hemicelluloses ou gommes ont une signification spéciale pour le maltage.

Les téguments de l'orge, les fractions de l'embryon et de l'endosperme, montrent des différences très nettes dans leur composition hydrocarbonée. L'embryon contient plus de 80% du raffinose et du saccharose. La cellulose, qui est abondante dans les téguments et est présente dans l'embryon, est virtuellement absente dans les cellules de l'endosperme central, pendant que l'amidon est trouvé seulement dans l'endosperme. Il n'est pas encore possible de tirer des conclusions bien définies au sujet de la distribution des pentosanes et du β-glucane dans les parties de la graine, à cause de difficultés dans le fractionnement ou dans l'analyse.

Des changements dans la constitution hydrocarbonée de la graine pendant le maltage exercent une influence profonde sur les propriétés du malt fini. Parmi les sucres libres, c'est le saccharose qui forme le principal substrat respiratoire pendant les premiers stades du départ de la croissance. Le raffinose est consommé rapidement si le grain dispose d'oxygène. L'hémicellulose β-glucane, qui peut se trouver associée dans la paroi cellulaire de l'endosperme, est progressivement dégradée pendant le maltage, le noyau friable du malt n'en contenant pratiquement pas.

Le sort réservé aux pentosanes n'est pas encore bien compris. Les taux des divers pentosanes ne sont pas élevés dans l'orge non germée et ils n'augmentent pas considérablement pendant le maltage. Bien qu'on doive conclure que l'étendue de la dégradation des pentosanes est minime, l'enlèvement de quantités même limitées de pentosanes aux matières qui constituent les parois cellulaires, pourrait aisément favoriser leur dissolution subséquente. Une perte d'environ 16% d'amidon se produit dans l'orge au cours du maltage; les grains d'amidon deviennent plus petits, les chaînes externes de l'amylopectine deviennent plus courtes, et le degré de polymérisation de l'amylose baisse légèrement.

Une méthode utile pour faire un rapport entre la composition hydrocarbonée de l'orge et l'extrait du malt fini est l'analyse de "l'hydrate de carbone insoluble" de Bishop et Marx, qui évalue "l'hydrate de carbone complémentaire à l'extrait." Le recours à ce dosage et à l'équation découlant de la méthode s'avère utile dans l'appréciation de variétés d'orge peu connues.

Les expériences *in vitro* fournissant l'explication du comportement de composés spécifiques, chimiquement définis, ne suffisent pas pour expliquer le métabolisme hydrocarboné de la graine vivante, dans laquelle des composants non-hydrocarbonés pourraient très bien jouer le rôle de facteurs primordiaux. Le futur immédiat de la recherche sur les hydrates de carbone de l'orge peut dépendre de raffinements dans les moyens mis en oeuvre pour l'étude de ces composants non-hydrocarbonés.

[*Resumen*]

El metabolismo de los carbohidratos de la cebada en relación al malteo.

Los carbohidratos de la cebada constituyen un grupo de substancias de especial importancia en el malteo. Son la fuente de casi todo el extracto fermentable y actúan como sustrato respiratorio y fuente de energía para las síntesis enzimáticas; algunos de ellos pueden impedir una rápida y completa modificación.

Entre los carbohidratos del grano de la cebada están: los azúcares, los oligosacáridos, el almidón, las hemicelulosas y la celulosa cruda; todo ésto constituye aproximadamente el 75% de la substancia seca del grano, con otro 5% más o menos, que seguramente forman los pentosanos. Los fructosanos son materiales de reserva en los internodos de la espiga; se utilizan extensamente en las primeras semanas del desarrollo de la espiga. El almidón de la cebada es un carbohidrato conocido, aunque los procesos involucrados en la elaboración del gránulo amiláceo y en la transformación de la planta de la forma vegetativa a la fructífera, aún continúan obscuros. Algunos trabajos recientes sugieren que gran parte del almidón del endospermo se produce por fotosíntesis directamente en la espiga. La elucidación de las relaciones de la estructura de la cascarilla, los bordes, y la pared del ovario con el endospermo del grano, puede ser de gran valor para seleccionar nuevas variedades para un rendimiento potencial. Las hemicelulosas o gomas son de especial importancia en el malteo aún cuando estén en una relativamente pequeña proporción en los carbohidratos de la cebada.

La cascarilla de la cebada, el embrión y las porciones del endospermo muestran una marcada diferencia en su composición de carbohidratos.

El embrión contiene sobre un 80% de rafinosa y de sacarosa. La celulosa, que es abundante en la cascarilla y está presente en el embrión, está virtualmente ausente de las células centrales del endospermo, mientras que el almidón se encuentra únicamente en el endospermo. No se pueden sacar todavía conclusiones definitivas acerca de la distribución de los pentosanos y del β-glucán entre las partes del grano, debido a las dificultades del fraccionamiento o del análisis.

Los cambios en la constitución de los carbohidratos del grano durante el malteo tienen profundos efectos en las propiedades de la malta terminada. Entre los azúcares libre, la sacarosa forma el sustrato respiratorio principal durante las primeras etapas de la iniciación del crecimiento. La rafinosa se utiliza rápidamente si el grano dispone de oxígeno. La hemicelulosa β-glucán que puede asociarse con las paredes celulares del endospermo, se degrada progresivamente durante el malteo, quedando el grano de malta frágil prácticamente libre de ese carbohidrato. El destino de los pentosanos no se comprende bien todavía. Los niveles de varios pentosanos son bajos en la cebada sin germinar y no aumentan considerablemente durante el malteo. Aún cuando la intensidad de la degradación de los pentosanos puede concluirse que es mínima, el retiro de aún pequeñas cantidades de pentosanos del material que forma las paredes celulares, puede fácilmente permitir su subsecuente disolución. Ocurre una pérdida de cerca de 16% del almidón de la cebada durante el malteo; los gránulos amiláceos disminuyen en tamaño, las cadenas externas de la amilopectina se vuelven más cortas, y el grado de polimerización de la amilosa se reduce ligeramente.

Un método útil de relacionar la composición de los carbohidratos de la cebada con el extracto de la malta terminada es el análisis de los "Carbohidratos Insolubles" de Bishop y Marx, el cual estima "los carbohidratos complementarios al extracto." El uso de esta medida y la ecuación desarrollada de acuerdo con este método es de importancia en la evaluación de las variedades de cebada poco conocidas.

Los experimentos *in vitro* tendientes a conocer el comportamiento específico de componentes definidos químicamente, no son suficientes para explicar el metabolismo de los carbohidratos del grano vivo, en los cuales los acompañantes no carbohidratos pueden muy bien resultar ser factores que controlen el fenómeno. El futuro inmediato de las investigaciones de los carbohidratos de la cebada puede depender de los refinamientos de los medios de estudio de éstos acompañantes no carbohidratos.

[*Zusammenfassung*]

Der Kohlenhydratstoffwechsel der Gerste in seiner Beziehung zum Mälzen.

Die Kohlenhydrate der Gerste bilden eine Gruppe von Stoffen, denen beim Mälzen besondere Bedeutung zukommt. Sie sind das Ausgangsmaterial für nahezu den gesamten vergärbaren Extrakt, sie dienen als Substrat zur Veratmung und als Energiequelle für die Enzymsynthese; einige Kohlenhydrate können die schnelle und vollständige Auflösung verhindern.

Unter den Kohlenhydraten des Gerstenkornes finden sich Zucker, Oligosaccharide, Stärke, Hemizellulosen und Rohzellulose; diese machen annähernd 75% der Trockensubstanz des Kornes aus, während weitere etwa 5% mit grosser Wahrscheinlichkeit aus Pentosanen bestehen. Fructosane bilden das Reservematerial in den Internodien des Halmes; sie werden während der ersten Wochen der Ährenentwicklung ausgiebig verwertet. Die Gerstenstärke ist kein unbekanntes Kohlenhydrat, die Vorgänge jedoch, die sich bei der Ausbildung des Stärkekornes und bei der Umwandlung der Pflanze vom vegetativen Zustand in den der Samenbildung abspielen, sind noch nicht aufgehellt. Aus neueren Arbeiten geht hervor, dass ein Grossteil der Endospermstärke durch Photosynthese unmittelbar in der Ähre erzeugt wird. Die Aufklärung der Beziehung der Struktur von Spelze, Granne und Samenhaut zum Korninhalt kann bei der Selektion neuer Sorten zur Ertragssteigerung von Wert sein. Obwohl sie nur einen verhältnismassig kleinen Anteil der Gerstenkohlenhydrate bilden, kommt den Hemizellulosen oder Gummi beim Mälzen besondere Bedeutung zu.

Die Fraktionen von Gerstenspelze, Keimling und Mehlkörper zeigen auffallende Unterschiede hinsichtlich ihrer Kohlenhydratzusammensetzung. Der Keimling enthält über 80% an Raffinose und Saccharose. Zellulose, die in der Spelze reichlich vorkommt und im Keimling vohanden ist, fehlt im wesentlichen in den mittleren Zellen des Mehlkörpers, wohingegen Stärke nur im Mehlkörper gefunden wird. Hinsichtlich der Verteilung der Pentosane und des β-Glucans auf die einzelnen Teile des Kornes können auf Grund von Schwierigkeiten bei der Fraktionierung oder Analyse noch keine endgültigen Schlüsse gezogen werden.

Die Veränderungen in der Zusammensetzung der Kohlenhydrate des Kornes während des Mälzens haben weitreichende Einflüsse auf die Eigenschaften des fertigen Malzes. Von den freien Zuckern bildet die Saccharose während der frühen Stadien des Wachstumsbeginns das hauptsächliche Substrat zur Veratmung.

Die Raffinose wird schnell verbraucht, wenn dem Korn Sauerstoff zur Verfügung steht. Die Hemizellulose β-Glucan, die mit den Zellwänden des Mehlkörpers in Verbindung stehen kann, wird während des Mälzens progressiv abgebaut, so dass sie im mürben Malzkorn praktisch nicht vorkommt. Das Schicksal der Pentosane ist noch nicht vollständig aufgeklärt. Die Gehalte an den verschiedenen Pentosanen in unvermälzter Gerste sind niedrig und nehmen während des Mälzens auch nicht nennenswert zu. Obwohl geschlossen werden muss, dass das Ausmass des Pentosanabbaus äusserst gering ist, könnte die Entfernung selbst begrenzter Pentosanmengen aus dem Baumaterial der Zellwände deren nachfolgende Auflösung leicht ermöglichen. Während des Mälzens tritt ein Verlust von etwa 16% der Gerstenstärke ein; die Stärkekörner nehmen an Grösse ab, die äusseren Ketten des Amylopectins werden kürzer und der Polymerisationsgrad der Amylose verringert sich etwas.

Eine brauchbare Methode, die Zusammensetzung der Gerstenkohlenhydrate zum Extrakt des fertigen Malzes in Beziehung zu setzen, ist die Bestimmung der "unlöslichen Kohlenhydrate" nach Bishop und Marx, bei der die "im Extrakt nicht erfassten Kohlenhydrate" ermittelt werden. Die Anwendung dieser Analyse und der Gleichung, die im Zusammenhang mit der Methode entwickelt wurde, ist zur Beurteilung unbekannter Gerstensorten wertvoll.

In vitro-Untersuchungen, die zum Verständnis des Verhaltens spezifischer, chemisch definierter Komponenten beitragen, genügen nicht zur Erklärung des Kohlenhydratstoffwechsels des lebenden Kornes, bei dem sich Nichtkohlenhydratbegleitstoffe durchaus als regelnde Faktoren erweisen können. Die unmittelbare Zukunft der Gerstenkohlenhydratforschung kann abhängen von der Verfeinerung der Mittel zur Untersuchung dieser Nichtkohlenhydratbegleitstoffe.

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LIPID METABOLISM IN GERMINATING BARLEY
I. THE FATS

BY

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I. THE FATS

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Ungerminated barley contains approximately 2% of petroleum-ether extractable lipid, one-third of which is restricted to the embryo. Analysis of the fatty acids of barley lipid by reversed-phase chromatography has shown the presence of 52% of linoleic, 28% of oleic and 11% of palmitic acid; small amounts of stearic, myristic and linolenic acids are also found. There is a variable decline in fat during malting, and malt fat is considerably depleted in the unsaturated C_{18} acids, oleic and linoleic. The slight rise in free fatty acids between barley and malt does not suffice to account for the loss of esterified material, which is rapidly metabolized by the developing seedling.

Introduction

Although a considerable volume of work has been published on cereal lipids in general and on the fats of oats, wheat germ and maize in particular, problems relating exclusively to barley fat and its metabolism during malting have on the whole been little studied. This omission is not surprising for, until very recently, methods available for critical analysis of the variable components of fats—*viz.*, the fatty acids—were tedious and time-consuming and required the initial extraction of some 50 g. of fat—a formidable undertaking from a source often containing rather less than 2% of ether-soluble materials. Moreover, according to Hopkins & Krause,[8] virtually all of the malt fat is left in the spent grains and is thus of no great significance to the brewer, important though it may be with regard to the nutritive value of brewer's grains. Again, although the potential development of rancidity as a result of lipase action in oat and wheat products is of considerable technical importance, this particular storage hazard seems to be of little significance in malting barleys—suggesting, possibly, a rather low level of lipase activity in the stored grain. It has certainly been recognized that barley fats form a respiratory substrate during storage and in the early days of seedling growth,[10] but, apart from this rather academic point, the importance of fat in barley metabolism would seem to be slight.

In contrast to this point of view, however, Rainbow[12] has recently suggested that the types of fatty acid present in malting barley may be of considerable importance with regard to the ultimate biological stability of the beer—a suggestion which stems from the observation that 10 μg. of certain straight-chain fatty acids of the C_{12}–C_{20} series per litre of beer can inhibit growth of lactobacilli. Clearly, fatty acids in barley will assume antibiotic significance only if they pass from malt to wort and finally survive all stages of the brewing process; in this connection a knowledge both of the potentialities of barley and malt lipase and of the solubilities of the relevant fatty acids in wort and beer becomes essential. Incidentally, the presence of fatty acids in beer need not conflict with the earlier finding that fat is retained in the spent grains, for the order of concentration envisaged as important (10^{-6}% in beer) would certainly evade detection by all but the most refined analytical methods.

Plant lipids.—In addition to the true fats, ethereal extracts of plant tissues contain waxes, phospholipids, sterols, resins and terpenes. The last three together constitute the unsaponifiable residue, and in the present work they have been largely ignored—as indeed they generally have been by most plant biochemists. True fats are triglycerides of fatty acids, usually esterified on the basis of maximum heterogeneity of acid composition per molecule of fat, so that simple triglycerides occur only when one fatty acid is present in very great excess of all others. Acids from C_4 to C_{24} occur principally in fats while higher acids are mainly found in waxes; the lower acids (C_4–C_{16}) are steam-volatile

and have not been extensively recorded from seed fats, though some representatives of this group are found in kernels of *Elaeis*.[3] Quite the most abundant in nature are the C_{18} acids, oleic and stearic, and the C_{16} acid, palmitic. Oleic, an unsaturated C_{18} acid (octadeca-9-enoic) accounts for not less than 40% of all naturally-occurring fatty acids, and there are indications that it may participate more actively in general metabolism than do some of the other acids.[4]

Although a wide variety of fatty acids is found in seed fats, the mixtures present in different genera of a family are usually qualitatively, and sometimes quantitatively, similar. In the Gramineae, oleic, linoleic (octadeca-9-12-dienoic) and palmitic (*n*-hexadecanoic) may together account for 95% of the total fatty acids.[7]

Fats are traditionally separated from seeds by soxhlet extraction, though recently simple percolation of ether through a column of finely-divided grain[19] has been employed with apparent success. After drying the fat, determination can be made of saponification value, which gives a measure of average chain lengths of the constituent fatty acids; iodine number, which indicates the extent of unsaturation; acid value, which allows determination of the proportion of free fatty acids; specific gravity; refractive index; and amount of unsaponifiable materials present. These data are useful for comparing different fat samples, but they do not of themselves go far towards characterizing any given sample. For fuller analysis, earlier methods relied on separation of the saponified material into saturated and unsaturated acids via the lead salts, followed by fractional distillation of the methyl esters and examination of the many possible separate fractions. More recently, low-temperature crystallization has proved successful in fractionating mixtures of fatty acids; but again, as with the lead salt method, discrete fractions each containing one molecular species are rarely obtained. These methods (which are fully described by Hilditch[7]) have now been largely superseded by two important new techniques—reverse-phase chromatography and gas chromatography.

Chromatographic techniques. — Reverse-phase chromatography depends on the partitioning of the mixture of fatty acids between two liquid phases—a tightly-absorbed liquid held stationary on a solid (filter paper or some type of column packing) and a mobile liquid which is more polar than the stationary phase. Many different pairs of liquids have been investigated, and Ballance & Crombie,[1] for example, found that for paper chromatography of C_{12}–C_{20} fatty acids the best results were obtained with liquid paraffin : aqueous acetic acid. The separated acids can be revealed on paper in the form of their silver or copper ferrocyanide complexes[18] or as mercury *s*-diphenylcarboxide complexes.[2]

For column chromatography kieselguhr is usually preferred though silica gel[11] has been successfully used. The kieselguhr, which must be unwettable, is impregnated with liquid paraffin or *cyclo*hexane, and elution is by means of one of the more polar solvents used in paper chromatography, *viz.*, acetone, acetic acid, methanol or ethanol. Clearly, many different combinations of stationary phase and eluant are possible and it is desirable to explore the various possibilities and select the optimum combination for a given sample of fat. For quantitative determinations column chromatography is more suitable than paper, though methods have been proposed for the latter which involve forming copper salts of the separated acids and thereafter determining the copper polarographically. With the column, however, the eluted acids are simply determined titrimetrically.

When all the acids present are of the same type (*e.g.*, straight-chain saturated) R_F values are inversely proportional to the numbers of carbon atoms in the molecule, but, unfortunately, addition of one double bond to the molecule affects the R value to the same degree as removal of two carbon atoms. This leads to the formation of "critical pairs" of acids, *e.g.*, palmitic-oleic, myristic-linoleic and lauric-linolenic, which move as single units instead of separating into the individual acids. The problem posed by these critical pairs can be solved by preparing complementary chromatograms, one with the mixtures of saturated and unsaturated acids and a second in which the unsaturated acids have been brominated, hydrogenated or oxidized, so giving products which have high R_F values and are rapidly eluted from the column.

The most recent development in fatty acid analysis is the use of gas chromatography. The mixed acids are first converted to their methyl esters and then carried in a stream of argon or other suitable carrier gas over a

carefully-prepared suitably-impregnated column maintained at a temperature approaching 200° C., and the emerging acids are detected by thermal conductivity or other means and automatically recorded.

Gas chromatography requires only μg. quantities of material, compared with the mg. quantities needed for reverse-phase work, and a satisfactory analysis can be completed in a few hours. Moreover, critical pairs of acids are apparently not encountered in gas chromatography. If routine analysis of fatty acid mixtures is regularly required there can be little doubt that gas chromatography will be the preferred method; its major disadvantage lies in the cost of the equipment. The apparatus for reverse-phase chromatography can be easily constructed in the laboratory, and though results are available only after several days spent in repeated titration, these results appear to be as reliable as those obtained by gas chromatography.

Experimental

General methods adopted.—In order to obtain an overall picture of lipid metabolism in germinating barley, estimations were first made of ether-soluble materials present in different varieties of ungerminated grain and at various stages throughout malting. Fractionated samples of grain were examined to determine the location of lipids within the corns, and separate analyses were performed on the embryo (or seedling) and the remainder of the grain at intervals during commercial malting. The types of fatty acid present in barley fat and in malt fat were determined qualitatively and quantitatively, principally by means of reverse-phase column chromatography.

Extraction of lipids.—After exploring other procedures, the usual soxhlet method was eventually employed, with petroleum ether (b.p. approx. 50° C.) as extractant. The simplified percolation method proposed by West & Lautenbach[19] took longer to complete owing to the large volumes of solvent required and it gave variable results, possibly as a result of unevenness of grind and channelling of the ether. Preliminary treatment of the ground grain with boiling ethanol, which has been advocated by various workers, certainly gave slightly higher yields of total lipids, but the increases appeared to be mainly due to enhanced extraction of waxes. It is probable, however, that petroleum ether does not extract all fat from the ungerminated grain (see later) but the use of the traditional solvent at least had the advantage of affording results which could be directly compared with those of earlier workers.

Barley and malt samples were finely ground in a hammer mill; samples of germinating grain were first dried at 90° C. in a vacuum desiccator and then, after grinding, re-dried to constant weight. The hazards of this type of drying are numerous, both enzymic and chemical, but no other method attempted gave satisfactorily consistent results. Reproducibility of the results of soxhlet extraction was good, with replicates all agreeing to within ±1·2% of the mean.

TABLE I

Barley Lipids
(% of dry grain)

Variety	Lipid, %
Herta, 1958	2·18
Plumage-Archer, 1958	2·00
Proctor, 1958	1·96
Proctor, 1959	2·01
Proctor, 1959	1·76
Mean	1·98

TABLE II

Distribution of Fat in Barley Grain*
(Fractions from pearling machine)

Principal components of fractions	% of grain	% of fat in fraction	% contribution to total fat
Husk	9·0	2·60	10
Husk + aleurone + embryo	4·7	6·85	14
Embryo + aleurone ..	9·6	6·74	28
Scutellum + aleurone + flour	10·2	4·24	18
Flour + furrow tissues ..	13·6	2·02	12
Endosperm	52·0	0·74	18

* Fat content of whole grain, 2·01%; sum of individual fractions, 2·32% of whole grain.

Lipids of ungerminated grain.—The total ether-soluble materials recovered from five different samples of barley are shown in Table I. These results are in agreement with previous findings by Sedlmeyer[14] and by Taufel & Rusch[17] who found, respectively, 2·0% and 1·8% of lipids in barley. Fractions of grain which had been treated in an experimental pearling machine were examined individually for lipid content, with the results

shown in Table II, where it will be seen that lipids are principally concentrated in the fractions which are richest in embryonic tissues. As separation by pearling does not give botanically homogeneous fractions,

TABLE III

DISTRIBUTION OF FAT WITHIN THE BARLEY GRAIN

(Embryos excised from 500 grains; all samples were of Proctor barley)

Origin of grain	Embryo fat		Fat in remainder of grain		Embryo fat as % of total
	Total (g.)	% dry weight	Total (g.)	% dry weight	
Berwickshire	0·100	15·4	0·238	1·5	29·6
Sandringham	0·116	16·4	0·236	1·2	32·9
Unknown ..	0·093	14·1	0·242	1·5	27·7
Means ..	0·103	15·3	0·238	1·4	30·0

examination was also made of the fat contents of excised embryos and of the residual embryo-free grain; results are given in Table III. It is interesting to note that nearly one-third of the lipid is restricted to the embryo, which itself represents less than 3% of the grain.

Changes in lipid content during germination.—In view of the predominance of fatty material in the embryo, it was considered desirable to perform separate analyses of the growing seedling and the residual endosperm and husk. In preliminary laboratory tests, Proctor barley was steeped at room temperature and maintained in aerated petri dishes on damp filter paper at 21° C. Growth was uniform and the corns remained free from moulds. Samples of 500 corns were removed at daily intervals and dissected into seedling and residue, both of which were dried and examined for lipid content.

Through the courtesy of a local brewery, samples of Proctor barley were obtained after a 2-day steep and again after 1, 3, 5 and 8 days on the malting floor. This malting, which was carried out specifically for the present study, was described by the maltster as "stubborn" and growth was slow, though at 8 days the malt appeared to be reasonably well modified. Again the samples were separated into seedling and endosperm + husk and analysed in the usual manner. Results of these two sets of analyses are given in Fig. 1.

One surprising feature of these analytical results is the apparent increase in ether-soluble material in the early days on the floor —an increase which was regularly recorded in similar determinations not reported here. The increase in lipids was especially marked in the non-living part of the grain, and it is highly improbable that fat synthesis is occurring at this time or in this tissue. It is, however, quite possible that in the ungerminated grain lipid is retained in fragments which initially are not easily penetrated by petroleum ether, but which are physically altered during steeping and growth and ultimately prove amenable to ether extraction. This point will be discussed more fully in a later Communication but, for the present, the lower yield of lipid from ungerminated grain compared with partly-grown malt is attributed to inadequate solvent penetration rather than to any metabolic change. In passing, it may be suggested that similar

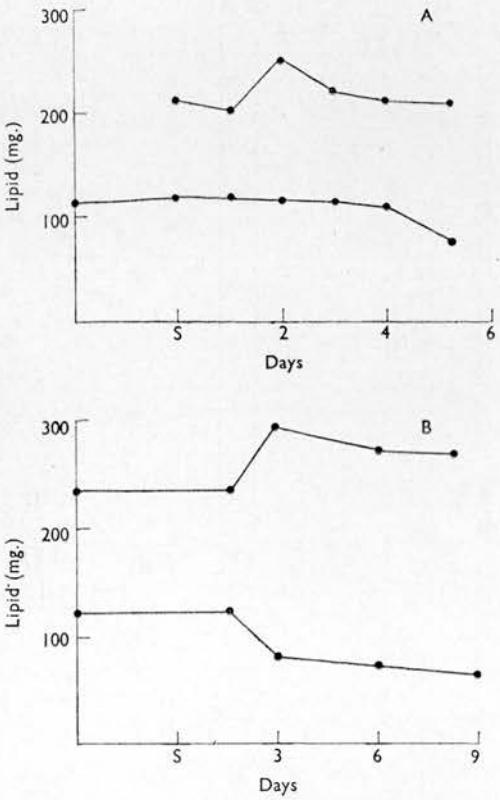

Fig. 1.—Changes in quantities of lipids extracted from germinating barley. A: laboratory-grown at 21° C. B: barley from maltings. In both A and B the upper line indicates lipid from endosperm + husk; the lower line indicates lipid from embryo or seedling; S = end of steep.

difficulties of solvent penetration could account for the discrepancy noted (Table II) between the results obtained for lipid content of an unfractionated sample of barley and the sum of the individual yields of the finely-abraded fractions prepared by pearling.

In spite of this extraction anomaly, it is clear that there is a marked, though variable, decline in the total lipids of the embryo as it develops to a seedling, and there would appear to be little doubt that fat is metabolized to a greater or lesser extent in the course of malting. No firm conclusions can as yet be drawn regarding the fate of the endosperm fat, and, moreover, in view of the extraction difficulties, the figures available for lipid contents of barleys and their malts must be regarded with reserve.

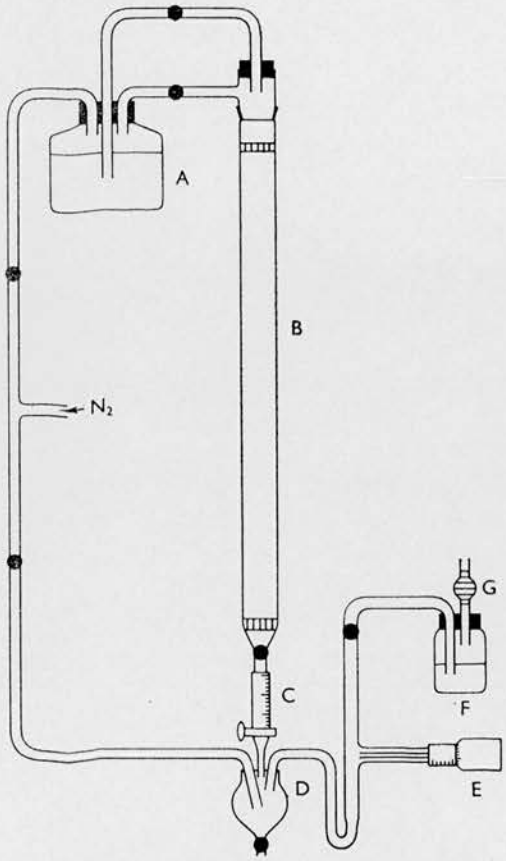

Fig. 2.—Apparatus used in reverse-phase chromatography. A, acetone reservoir; B, column of treated kieselguhr; C, 2-ml. measuring device; D, titration cell; E, *Agla* micro-burette; F, alkali reservoir; G, CO_2 absorption tube. The solid black circles indicate the positions of stopcocks.

Chromatographic separation of fatty acids: reverse-phase column chromatography. — Samples of crude barley or malt fat were dissolved in ethanolic KOH and refluxed for 1 hr. After removal of the ethanol and addition of water, the unsaponifiable material was transferred to ether and separated from the aqueous phase. Free fatty acids were recovered from their soaps by acidification and extraction in ether and the mixture of free fatty acids was used for chromatographic separation in the apparatus shown in Fig. 2.

Various methods were investigated for preparing and packing the column, and the most successful, which was essentially that of Silk & Hahn,[15] was as follows: 300 g. of *Celite* 545 were stirred into 4 litres of water and allowed to stand undisturbed for 1 hr. Suspended particles were decanted off, the process was repeated, and the final residue was dried at 110° C., cooled and exposed to the vapour of dichlorodimethylsilane in a partially-evacuated desiccator overnight. The kieselguhr was washed free of acids with methanol and re-dried; the treated material was completely non-wettable.

Treated kieselguhr (93 g.) was suspended in a solution of liquid paraffin* (75 ml.) (which had previously been equilibrated with acetone) and ether (250 ml.). The ether was evaporated off until a coarse homogeneous powder was obtained; this was dried for 2 hr. in a vacuum oven. 30 g. of this powder was thoroughly mixed in a top-driven macerator with 83% aqueous acetone and the slurry was transferred to a separating funnel with a wide-bore stopcock. All air was expelled by opening the stopcock and boiling the slurry in a water-bath and, after closing the stopcock and cooling to room temperature, the stem of the funnel was inserted into the top of the column, which was full of air-free 83% acetone. The column could now be filled with the mull without introduction of air, and judicious tapping and use of the plunger in the column gave homogeneity of packing. This technique of column-filling has been described in detail, because unsuccessful packing was the biggest single early cause of failure in subsequent operations.

To load the column, the fatty acid mixture was dissolved in paraffin with gentle heating, using 2·1 ml. of paraffin to each 7 mg. of

* *Nujol.*

mixed acids; a total of approx. 100 mg. of fatty acids was separated at a time. The loaded paraffin was then added to 15 ml. of ether, non-wetting kieselguhr (0·26 g. for each 0·2 ml. of paraffin) was added, the ether was removed by evaporation and the powdery mull was dried at 60° C. for 12 hr. in a vacuum oven. Finally, the mull was slurried with 15 ml. of 55% aqueous acetone, boiled to expel air, cooled and transferred to the column. After settling, the mull was gently tamped down and covered with a band of air-free kieselguhr in acetone.

Aqueous acetone was used as eluant, at concentrations ranging from 55% to 85% (v/v) and containing bromothymol blue indicator. The acetone solutions were equilibrated with liquid paraffin and clarified by filtration under pressure through a 3-cm. bed of kieselguhr-paraffin mull.

In operating the column (Fig. 2) nitrogen was used to transfer acetone of the desired strength from the reservoir (A) to the top of the kieselguhr (B) and, by suitable manipulation of stopcocks, pressures were controlled and conditions adjusted to give a flow rate of not more than 40 ml. per hr. The 2-ml. measuring device (C) was constructed from a micro-burette, the titration cell (D) was a small dropping funnel whose contents were stirred by nitrogen and the 0·01-N KOH was delivered and measured by an *Agla* micrometer syringe (E) which could be filled directly from a reservoir (F) without removing it from the apparatus. The column was operated at more or less constant temperature; if wide fluctuations in ambient temperatures are experienced, then water-jacketing is essential.

Several separations of known mixtures of fatty acids were carried out before attempts were made to examine barley fat; results of a typical analysis of a known mixture are given in Table IV.

TABLE IV

RECOVERY OF KNOWN ACIDS FROM COLUMN

Acid	Known mixture	Separated on column
Lauric ..	86·2	84·8
Myristic ..	3·5	3·7
Palmitic ..	10·3	11·5

Oxidation technique.—Since "critical pairs" of saturated and unsaturated fatty acids are common in natural fats, it was essential to determine values separately for the unsaturated members of the pairs. To this end, the unsaturated components were oxidized with alkaline permanganate at room temperature by the method of Crombie & Boatman,[3] and a second passage through the column now gave values for the saturated acids alone; unsaturated acids were found by difference. This method gave clear-cut separations without any obvious tailing of the degradation products.

A total of 300–400 ml. of acetone was required to elute all the fatty acids; oxidation products and linolenic were successively removed by 55% acetone, myristic and linoleic by 60%, palmitic and oleic by 65%, stearic by 70% and any higher acids by 75%. With each successive concentration of acetone, the titres of the 2-ml. aliquots rose to a maximum and then subsided almost to zero so that plotting of the titration figures gave the familiar peak and trough type of diagram; concentrations of the acids were determined from the sums of the individual titres at the appropriate concentrations of acetone.

Results of analyses of fatty acids prepared from Proctor (1959) barley and from its malt are given in Table V. Other samples of barley gave generally similar results, though it may be noted that when the column was

TABLE V

FATTY ACID COMPONENTS OF BARLEY AND MALT LIPIDS

Fatty acid	Barley (% of total fatty acids)	Malt (% of total fatty acids)
n-tetradecanoic C_{14}—myristic	1·0	0·4
n-hexadecanoic C_{16}—palmitic	11·5	25·2
n-octadecanoic C_{18}—stearic	3·1	5·5
octadeca-9-enoic C_{18}—oleic	28·0	13·4
octadeca-9.12-dienoic C_{18}—linoleic	52·3	51·5
octadeca-9.12.15-trienoic C_{18}—linolenic	4·1	4·0
Total lipid extracted ..	2·77 g. per 5,000 corns	1·57 g. per 5,000 corns
Iodine value of fatty acid mixture	128·2	124·6

loaded by the method of Howard & Martin[9] (*i.e.*, after dissolution of the acids in 55% acetone) no stearic acid was detected, presumably owing to the low solubility of the C_{18} saturated material.

From a knowledge of the total weights of fat in barley and in its malt combined with analyses of the two fats into their component fatty acids, it should be possible to compile absolute figures for changes in individual acids during malting. However, as has been shown earlier, it is doubtful whether the usual extraction method successfully removes all fat from dry grain, and any such calculations must therefore be treated with very great caution. It is clear, nevertheless, that approximately 75% of the oleic and 40% of each of the linoleic and linolenic acids detected in the original barley fat are missing from the corresponding malt fat.

Free fatty acids and unsaponifiable residue.—In addition to the observed decline in total lipid and the relatively greater loss of unsaturated acids compared with saturated, it is possible that changes may be taking place in the extent of esterification—*i.e.*, in the proportions of free fatty acids present in the crude lipid. To investigate this possibility, 5,000-corn samples of barley were obtained at different stages in malting, *viz.*, as ungerminated grain, as 2-day and 5-day malt, and as 9-day malt just prior to kilning. Lipids were extracted in the usual manner and the crude lipid was dissolved in hot ethanol which had previously been adjusted to a phenolphthalein end-point (pH 9·4). The total acidity was determined by titrating the solution back to pH 9·4, and free fatty acids were removed by partitioning between light petroleum and dilute sodium carbonate solution. Free fatty acids were removed from the aqueous extract and estimated titrimetrically. Amounts of total fatty acids and of unsaponifiable residues were determined for the light petroleum extracts. Results of these analyses are given in Table VI. No figures are presented for the analyses performed on the ungerminated grain, for once again the total amount of fat extracted was significantly less than that obtained at 2 days of growth and comparisons may therefore be misleading. There is, of course, no guarantee that *all* the lipid is extracted even from the growing malt; the best that can be said is that these figures are not obviously anomalous.

TABLE VI

FREE FATTY ACIDS AND UNSAPONIFIABLE RESIDUES
(mg. per 5,000 corns; *i.e.*, 168·6 g. original dry grain)

	2-day malt	4-day malt	9-day malt
Total lipid	3,167	2,835	1,583
Unsaponifiables ..	98	97	99
Free fatty acids* ..	20	21	45
Esterified fatty acids	2,728	2,414	1,209
Fatty acid as % fat†	86	85	80

* As linoleic.

† Fat taken as: crude lipid—(unsaponifiables + free acids).

100 g. of glycerol tristearate will yield 88 g. of C_{18} acid on saponification; it is clear from the results quoted in the bottom line of Table VI that virtually all of the fat in the 2-day and 4-day malts can be satisfactorily accounted for in terms of glycerol and fatty acid. The discrepancy apparent in the 9-day malt may reflect the presence of considerable proportions of glycerol molecules which are only partially esterified with long-chain fatty acids. No evidence was found for the presence in malt of shorter-chain acids than those originally present in barley (see Table IV).

The acid value of the extracted lipids rose from 8·8 in barley and 2-day malt to 42·5 in 9-day malt—an initial value and an increase which are both much too great to be accounted for by the recorded increases in free fatty acids. A similar discrepancy between acid values of extracted lipids and total free fatty acids has been noted by Crombie & Comber[4] in germinating *Citrullus* seeds; they tentatively suggest that the discrepancy may be due to titration of a phosphatide with alkali-binding properties. For the present, we merely note the anomaly and leave it unexplained.

Paper chromatography.—Although reverse-phase column chromatography has proved a completely successful method for characterizing fatty acids from barley, paper chromatography of these materials has, in our hands, proved to be of very limited value. No great difficulty was experienced in separating mixtures of known saturated acids on paper impregnated with *Nujol* or undecane; but

linoleic acid caused extensive streaking unless it was present in very low concentration. As linoleic acid is a major constituent of barley fat, its awkward behaviour introduced considerable difficulties in manipulation, and no sustained attempt was made to develop reverse-phase paper chromatography on a quantitative basis.

Discussion

The amounts of lipid extracted from barley by petroleum ether and the proportionate composition of the constituent fatty acids, as determined by reverse-phase chromatography, are in broad general agreement with results of earlier analyses by Taufel & Rusch[17]; myristic acid, however, is now reported, apparently for the first time, from barley and malt fat. The presence of this C_{14} saturated acid was confirmed by Dr. Lough, of the Rowatt Research Institute, who kindly analysed samples of barley and malt fatty acids by gas chromatography, and found the same general picture of composition as had been determined by the column technique.

Barley fat is characterized by outstandingly high proportions of di- and mono-unsaturated C_{18} fatty acids—oleic and linoleic—which between them account for 80% of the total extracted fatty acids. Not surprisingly, barley fat is remarkably similar to wheat germ oil which, according to Sullivan & Bailey,[16] also contains 52% of linoleic, 28% of oleic and 11% of palmitic acid. These figures are identical with those found for barley fatty acid components; wheat germ differs in containing small amounts of lignoceric acid (C_{24}, saturated) and in apparently lacking myristic acid.

Although there is good agreement regarding the amounts, composition and location of barley fat, with a pleasing similarity apparent between the acid composition of barley and that of its close relative, wheat, there is much less apparent agreement about the extent of metabolic changes in fat during malting. Thus, for example, Deleano[6] found that the total amount of fat remained unchanged until the 8th day on the malting floor; thereafter it declined rapidly. Sedlmeyer[14] recorded a loss of 30% of the original barley fat and Taufel & Rusch[17] a loss of 12%. In the present investigation, we have observed losses of fat ranging from a mere 4% to a maximum of 40%. There is no reason to suppose that any of these values are grossly in error (except, possibly, the initial figure for total barley lipid): rather it seems probable that the extent of fat metabolism may vary dramatically with different samples of barley malted under different conditions. To illustrate the possible extent of variation, compare the changes observed in embryo fat during 2 days of steeping (Fig. 1) with the changes found when excised embryos are maintained on moist filter paper (Table VII). In the steep, sucrose disappears from the embryo while lipids persist; in aerobic isolation, not only is sucrose metabolized but lipid reserves are depleted to a level resembling that of mature leaf tissue—possibly to the lowest level necessary for structural identity of mitochondria and cytoplasmic membranes. Barley, then, *can* rapidly metabolize fat; the extent to which it actually does so must depend on malting conditions. It may be suggested, however, that it might be economically worth-while to know more of the conditions in which fat does serve as a respiratory substrate in the embryo during malting, for malting loss at the expense of fat would surely be a gain in terms of carbohydrate and, ultimately, extract.

TABLE VII

Lipid Metabolism in Isolated Embryos
(mg. lipid per 500 embryos)

Time of incubation (hr.)	Lipid content*	
3	122	102
48	27	19
72	—	18

* Both samples were of Proctor barley.

Although the facts reported here give an adequate background for a more detailed examination of enzymes involved in lipid metabolism, one technical problem remains as yet unsolved—*viz.*, the best means of securing complete extraction of lipid from ungerminated grain. Analytically the "extra" fat extractable from 2-day malt appears to be identical with that initially obtained from dry grain; its reluctance to emerge from finely-ground grain may be due to its location within, for example, the thick-walled aleurone cells which are not readily destroyed on grinding, or the peculiar

suberized cells of the residual tissues of the furrow. Permeability changes known to take place in the outer layers of the caryopsis when the grain is steeped may also be involved, but the true explanation of this awkward behaviour of the fat still remains unknown.

The mechanisms involved in lipid metabolism in plants are obscure and have been remarkably little studied. There is no quantitative change in the unsaponifiable fraction, which, as both Taufel & Rusch[17] and Sedlmeyer[14] have already shown, remains constant throughout malting; qualitative modification of this material is, of course, not precluded. Changes which occur in the lipid are thus at the expense of the true fat, with the unsaturated acids apparently of special importance. No short-chain fatty acids have been detected in malt and, although there is an increase in free fatty acids, this increase is not commensurate with the loss of esterified fatty acids (Table VI); these two facts suggest that the presumed products of lipase activity are rapidly metabolized. James[10] has provided evidence which suggests that fats not only contribute to barley respiration but also are directly implicated in the formation of cutin in the developing acrospire. The problems surrounding mobilization of barley fats are thus complex; lipase is certainly implicated and the potentialities of this hydrolytic system in malting will form the subject of Part II of this series of Communications.

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XI

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LIPID METABOLISM IN GERMINATING BARLEY
II. BARLEY LIPASE

BY

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H. B. WHITE, F.H-W.C., A.R.I.C.

COMMUNICATIONS

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II. BARLEY LIPASE

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Although lipase activity, as estimated by hydrolysis of triolein or tributyrin, can be detected in ground barley, not more than 15% of the lipase can be readily extracted from the grain either before germination or during malting. Over two-thirds of the total lipase content of the grain is found in the embryo and most of the remainder is concentrated in the aleurone layer. Both total and extractable lipase activities diminish during steeping and then increase slowly during growth on the malting floor. The fraction of the lipase which can be brought into solution has a sharp pH optimum at 6·8 and is extremely susceptible to inactivation by heat.

Introduction

Although the existence of plant esterases was established by Green[6] in 1891, there have been remarkably few satisfactory quantitative studies of this group of enzymes. Studies of lipase in particular have largely been restricted to enzymes of animal origin, though lipases of *Ricinus* (castor oil)[8] and to a lesser extent *Helianthus* (sunflower) seeds have attracted some attention owing to the fact that fatty materials form major food reserves in the endosperm of *Ricinus* and in the cotyledons of *Helianthus*. However, not only is *Ricinus* lipase apparently atypical in respect of pH relationships[8] but it is also virtually insoluble in water, so that estimations of activity must be based on the use of finely-ground powders prepared from the ether-insoluble residues of the endosperm, and precise kinetic data are difficult to establish. Indeed, this question of solubility has formed a major barrier to full exploration of the activities of plant lipases.

As far as the cereals are concerned, it appears that a wheat germ lipase may be obtained in a water-soluble form,[13] though oat lipase resists extraction[7] and there is some doubt regarding barley lipase. Thus, Maestrini[10] has recorded the presence of active but water-insoluble lipase in germinating barley, while van Laer[14] has demonstrated lipase activity in malt extract —a finding which indicates at least a partial degree of water-solubility in the malt enzyme.

In view of the very limited amount of sustained work which has been reported with barley and malt lipases, it seemed desirable first to survey methods of estimating barley lipase in tissue breis before exploring possible techniques of extracting the enzyme, or enzymes. There is good evidence for the presence of lipase in barley: indeed, in the first part of this investigation[9] it was shown that isolated embryos can metabolize their reserves of fat almost completely within 48 hr. in starvation conditions. The problem now is to attempt to make some quantitative assessment of the behaviour of barley lipase.

Although originally this work was undertaken simply with a view to extending knowledge of an aspect of barley metabolism which in the past has been rather neglected, recent developments in gas chromatographic analysis have suggested that understanding of the metabolism of triglycerides and of fatty acids of barley during malting may eventually be of practical importance in that traces of materials derived from lipids[1] may offer a significant contribution to the complex mixture of trace substances affecting the organoleptic qualities of beer.

Experimental

Numerous methods have been proposed for determining lipase activity either in tissue breis or in aqueous solutions. The most frequently used procedures are variations on the titrimetric method of Willstätter *et al.*,[15] though techniques involving stalagmometry, nephelometry, manometry or colorimetric estimation of the glycerol liberated instead of the free fatty acids have been proposed and used with greater or lesser degrees of success. However, since none of these variants appeared to have any pronounced advantage over the usual acidimetric method, techniques used in the present study have largely been restricted to the determination by titration of fatty acids liberated from a suitable substrate. All the results reported here were obtained with a sample of Proctor barley from the 1959 harvest.

Lipase activity of ground grain.—The first method investigated for estimating total lipase was that of Sullivan & Howe[13] and, briefly, involved incubation of ground grain with triolein in 10 ml. of water for 24 hr. as a prelude to extraction and estimation of liberated fatty acids. Difficulties were experienced in detecting end points with any degree of confidence in the very turbid final extracts and, although these difficulties were largely overcome by heating before titration, replicate determinations were variable and titre differences were extremely low. It may be noted that wheat germ, for which the method was devised, contains water-soluble lipase whereas some doubt exists about the extractability of barley lipase: the low titre differences found with barley as enzyme source may thus have been due to an innate low level of lipase, but they could equally well have resulted from failure to secure contact between substrate and enzyme within the ground grain.

Greater success was achieved with the method proposed by Hutchison & Martin[7] for oat lipase—a method which differs from that of Sullivan & Howe by specifying a very fine grind of grain and a restricted supply of water. The fine grind was obtained by using a hammer mill and, initially, 4-ml. portions of water were added to 5 g. of ground grain + triolein: after incubation and extraction of fatty acid (see below), titrations were carried out against 0·01-N ethanolic potassium hydroxide. Although replicate determinations gave consistent results, the controls in which lipase had been inactivated by heating for 1 hr. at 100° C. gave high titration values and, as a preliminary, therefore, free fatty acids were removed by extracting the ground material with 3 × 5 vol. of petroleum ether at room temperature and then drying the residue till free from ether. This procedure had no detectable effect on lipase activity and it allowed use of controls containing only traces of free fatty acids.

However, although additions to 5-g. portions of barley + triolein of a commercial preparation of lipase, in amounts ranging from 0·1 to 1·0 g., gave proportionately increasing values for enzyme activity, additions of increasing amounts of ground barley to a fixed volume of emulsion did not give the expected regular increases in amounts of free fatty acid liberated (Fig. 1). This discrepancy suggested that barley lipase activity might be strongly dependent on the amount of moisture in the reaction mixture; in support of this was the observation that in a series of determinations carried out with different amounts of ground barley, but with each reaction mixture adjusted to constant moisture content, consistent results were obtained per g. of barley used in the range 0·5–5·0 g. of barley (Fig. 1). The level of moisture found to give maximum values for

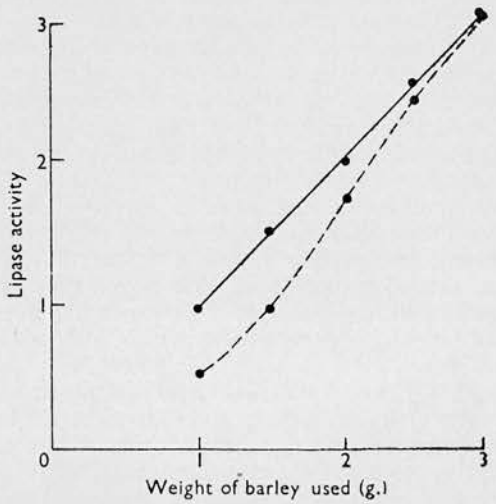

Fig. 1.—Effect of moisture on lipase activity of ground grain. Solid line: water content adjusted to 40%; broken line: constant volume of water added to each sample.

lipase activity was 40%. This figure approximates to the moisture content of barley during malting and it proved to give satisfactory results at all stages of growth.

The method finally adopted for estimation of total lipase activity in ground barley was therefore as follows: A sample of finely-ground ether-extracted barley was placed in a $2\frac{1}{2} \times \frac{1}{2}$-in. test tube, sufficient water was added to bring the moisture content to 40% and then 0·1 ml. of triolein, or olive oil, was mixed in thoroughly and the tube was incubated at 37° C. for 24 hr. The unsaponified fat and the free fatty acids were then extracted in petroleum ether, the ether was removed and the fatty acids were transferred to 50:50 (v/v) ethanol:benzene and titrated against 0·01-N ethanolic KOH. With material which had been steeped or grown, either in the laboratory or in commercial maltings, the grains were first ground roughly in a chilled hammer mill and then partially dried by treatment with 5 vol. of cold acetone. A second milling gave a uniform fine grist in which lipase activity could be estimated in the same manner as was used with ungerminated grain. Comparative studies carried out with untreated grist and with a grist which had been acetone-dried indicated that the drying process had no apparent deleterious effect on lipase, provided all materials were kept cool. Lipase activity has normally been expressed in terms of titration values per g. of grain, *i.e.*, as titre differences obtained using 0·01-N ethanolic KOH to estimate fatty acid production from triolein during 24 hr. at 37° C. With the sample of Proctor barley used in the investigation, 9 separate estimations gave a mean value of 1·01 with a standard deviation of ±0·03; other samples of Proctor barley gave results of a similar order of magnitude.

Distribution of lipase within the barley grain.—Fractions obtained from an experimental pearling machine were examined for lipase activity by the method described above. The fractions richest in active lipase were those containing embryo and aleurone, and from the results even of this rather crude fractionation it could be seen that more than 55% of the total grain lipase was concentrated in the peripheral 25% of the corn. A more accurate estimate of the proportion of lipase present in the embryo was obtained by analysing grain which had been separated by hand into embryo and endosperm; results of these analyses, shown in Table I, indicate that in fact two-thirds of the lipase is located in the embryo. Although excision of a sufficient number of embryos to allow chemical analysis is rather a tedious process compared with mechanical pearling, it does give clean separation of two of the principal tissues of the corn. Unfortunately no reliable method has yet been found for separating large quantities of aleurone from the predominantly starchy endosperm, and, to determine at least qualitatively the location of endospermic lipase, use was made of the histochemical technique of George & Iype.[5] This method depends on hydrolysis of *Tween* 80 (polyoxyethylene sorbitan mono-oleate) by lipase: a calcium soap is then formed *in situ* and subsequent treatment successively with lead nitrate, yellow ammonium sulphide and acetic acid reveals sites of lipase activity as dark brown deposits in the cells.

In transverse sections cut through the endosperm, lipase was seen to be concentrated in the aleurone, both in the main body of the 3-layered aleurone and in the scattered single aleurone cells which dip down into the furrow of the grain; a few isolated brown granules suggested the presence of a limited

TABLE I

DISTRIBUTION OF LIPASE WITHIN THE GRAIN

Grain fraction	% of grain (as excised)	Lipase activity* (per g.)	% of total activity in fraction
Embryo	4	17·0	68
Endosperm (including aleurone)	96	0·33	32
Whole grain (undissected)	—	1·06	—
Whole grain (sum of fractions)	—	1·00	—

* See text.

amount of lipase in the sub-aleurone layer, but the greater part of the endosperm appeared to be devoid of lipase activity. Tests carried out on sections cut longitudinally through the embryo confirmed the results of the gross analysis and showed that this part of the grain was indeed rich in lipase; it further appeared that, within the embryo, the scutellum (particularly its epithelial layer) was rich in lipase with rootlets and coleoptile exhibiting rather restricted activity.

Changes in lipase activity during germination.—Barley was steeped at room temperature for 48 hr. and then grown at 21° C. for 5 days and lipase activity was determined in aliquots of ground grain at daily intervals. Results of a typical set of analyses are shown in Fig. 2, where it can be seen that

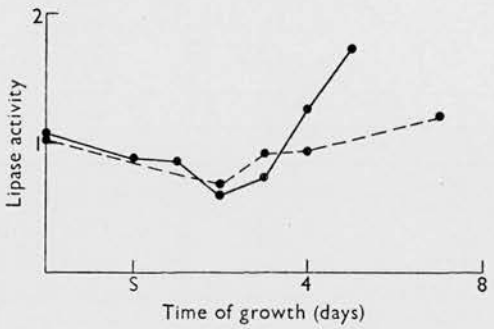

Fig. 2.—Lipase activity during germination. S: end of steep. Solid line: aerobic growth at 20° C.; broken line: commercial malting.

there was an early decline in enzyme activity followed by an increase which, in this experiment, gave a final level of lipase activity nearly twice as high as that detected in the original grain. Also shown in Fig. 2 are results of determinations made on samples obtained from a commercial maltings, where growth was at 16° C. Here the same general pattern was apparent, though the final rise in activity was not so pronounced.

From the results of this preliminary survey, it is clear that potentially active lipase is present in the embryo and aleurone layer and that during malting changes take place in the magnitude of lipase activity—changes which are not dissimilar to those known to occur with certain other hydrolytic enzymes. For more precise information about the potentialities of barley lipase, some separation of enzyme from the inert structural components of the grain was essential and, accordingly, attempts were made to secure active preparations of lipase in aqueous solution.

Estimation of activity of soluble lipase.—Working on the assumption that, as van Laer[14] has shown, at least some of the lipase of barley can be detected in aqueous extracts, methods of determining activity of solutions of lipase were examined, using both barley and malt as starting materials. Two of the more promising methods appeared to be those of Fiore & Nord[4] and of Singer & Hofstee.[11] Both techniques gave reproducible positive results both for barley and for malt, thus confirming van Laer's observations but, as the method of Singer & Hofstee could also be used for estimation of lipase in ground grain, it was preferred for routine use. In this method, 1 ml. of a 10% (v/v) emulsion of tributyrin, 2 ml. of 0·1-M phosphate buffer of the desired pH, an aliquot of enzyme solution (see below) and sufficient water to give 6 ml. were mixed and incubated for a convenient length of time (3–18 hr.). After incubation, enzyme activity was stopped by addition of 10% (w/w) phosphoric acid to pH 3. According to Singer & Hofstee, this pH is critical for wheat germ lipase: if excess acid is added, hydrolysis during the subsequent distillation produces extra butyric acid and, although this extra acid is detected in the control determinations made with inactivated lipase, it is still undesirable. On the other hand, if the final level is much higher than pH 3, recoveries of butyric acid are not complete. Similar conditions were found to obtain with lipases extracted from barley and malt, and care was therefore taken to ensure consistent attainment of pH 3 before distillation. After addition of water to 12 ml., the mixture was centrifuged and the supernatant was steam-distilled until 100 ml. of distillate was collected, in an atmosphere of nitrogen. Titration was carried out with 0·01-N alkali, also in presence of nitrogen, and results are again quoted as titre differences. Barley lipase was much more active towards tributyrin than towards triolein, and the results for extracted enzymes are thus not strictly comparable with those obtained with ground barley where triolein formed the substrate. Possible reasons for this difference will be discussed later.

Extraction of lipase.—1 kg. of barley was ground slowly in a cooled mill and extracted with 3 vol. of water or other solvent for 2 hr. with continuous stirring. The mixture was clarified by centrifugation and filtered through *Celite*. Ammonium sulphate was added to saturation and the resultant precipitate was separated, redissolved, dialysed against cold running water till free from sulphate, dissolved in the minimum volume of water, reprecipitated with 3 vol. of acetone and dried. Steeped grain was dried by treatment with acetone before being ground. Conditions of grinding examined included use of a coffee mill, a hammer mill at room temperature and a hammer mill maintained at 4° C.; different extractants used included water, 0·6% NaCl and 1% papain. The effects of these different procedures on yields of crude enzyme and on the total activities of the preparations are shown in Table II. The coffee mill was inefficient, possibly as a result of the rather large particle size of the final grist. Extraction was better from grists prepared in the hammer mill, especially when careful attention was paid to chilling the mill; however, it proved difficult to maintain consistent low temperatures and, since the improvement achieved by chilling amounted to an increase of less than 2% in terms of total activity, grists were prepared by slow grinding at room temperature. The extractant used had a more pronounced effect on yield of precipitate than it had on its lipase activity. Thus, treatment with papain allowed extraction of more than twice as much precipitable material from barley as did water, but the total activity of this precipitate was only 20% higher than that from the aqueous extract, and since, as will appear later, the maximum amount of lipase which could be removed from the grain did not exceed 15% of the total lipase, the advantage secured by using papain was not as great as would appear from the figures quoted in Table II. Again, although the preparation of papain used appeared to be devoid of lipase activity, we were reluctant to introduce as extractant an enzyme mixture foreign to barley unless it proved to be essential; it therefore seemed wiser to employ the solvent which gave the most active preparation per unit of product *viz.* water. Enzymes were therefore prepared by aqueous extraction from grain which had been slowly ground at a temperature not exceeding 18° C. in a hammer mill, and precipitated as described above by ammonium sulphate and acetone.

TABLE II

EXTRACTION OF LIPASE FROM BARLEY

Extraction data	Crude lipase		
	Yield (g. per kg.)	Activity* (per 10 mg.)	Total extractable activity
Method of grinding (water as extractant):			
Coffee mill (18° C.)	1·94	3·3	640
Hammer mill (room temperature)	1·97	3·7	730
Hammer mill (4° C.)	1·97	3·9	768
Extractant (hammer mill grind, ≯ 18° C.)			
Water	2·11	3·3	690
0·6% NaCl	2·56	2·7	691
1% papain	4·41	1·8	814

* See text.

Effect of pH on lipase activity.—For the range 5·8–8·0, 0·1-M NaH_2PO_4-Na_2HPO_4 buffer was incorporated in the incubation mixture; for values between pH 4·5 and 5·8 buffers were prepared by titrating 0·1-M phosphoric acid with 0·1-M sodium hydroxide to the desired pH level. Incubation was at 21° C. for 18 hr. and results of determinations carried out with crude enzyme preparations from barley and from malt are shown in Fig. 3, where it is clear that both show maximum activity at pH 6·8.

Effect of temperature on lipase activity.—2-ml. portions of enzyme solution containing

5 mg. of precipitated enzyme were incubated at pH 6·8 with tributyrin for 18 hr. at temperatures ranging from 16° to 37° C., and determinations of free acid were made

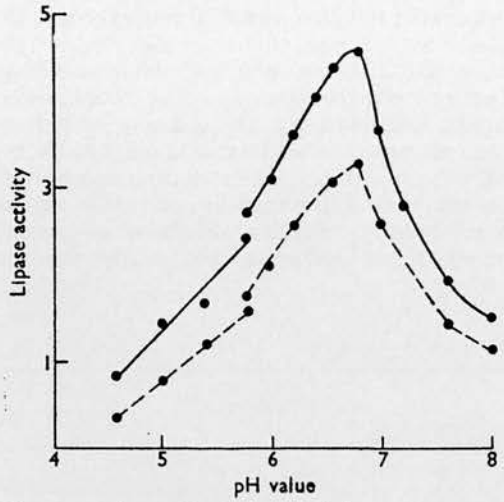

Fig. 3.—Effect of pH on activity of extracted lipase. Solid line: preparation from barley; broken line: preparation from malt.

in the usual manner. Results are shown in Fig. 4 (solid line) where the low apparent optimum temperature (28° C.) will be noted. When portions of enzyme solution were maintained at various temperatures for 6 hr. before addition of substrate and

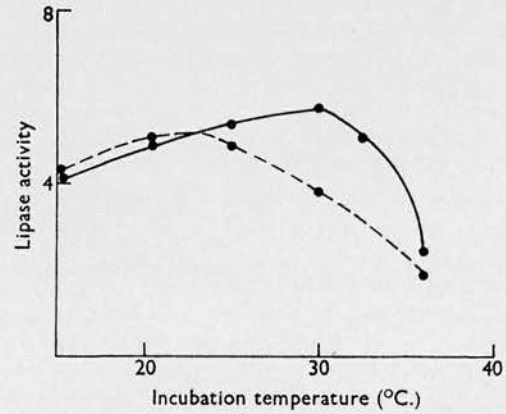

Fig. 4.—Effect of temperature on activity of extracted lipase. Solid line: enzyme and substrate incubated directly for 18 hr. at temperatures shown; broken line: enzyme preincubated in water for 6 hr. at temperatures shown, enzyme and substrate then added and incubation continued for 18 hr.

subsequent incubation, the apparent temperature optimum (Fig. 4, broken line) was even lower: it seems, therefore, that extracted lipase may be peculiarly susceptible to thermal inactivation. This drastic effect of temperature was apparent only with enzymes in solution, and with ground grain the apparent optimum for lipase activity in the experimental conditions used was 50° C.; however, at 60° C. only 10% of the maximal activity remained.

Extractability of lipase from barley.—Although it soon became clear that an active lipase could be extracted from barley into water, no assessment was possible of the proportion of the enzyme which was easily removed from the grain, as the methods developed for ground barley required a different substrate from that found to be most satisfactory with extracted enzyme. However, it was possible to use tributyrin as a substrate with ground grain and so to obtain some idea of the completeness of extraction achieved. In this determination, 1-g. samples of ground grain were incubated with 1 ml. of tributyrin and sufficient water to give 6 ml., and free fatty acids were estimated as usual after steam distillation. Results of determinations made on freshly-milled grain and on grain which had been extracted to give lipase solutions showed that 11–12% of the total lipase was regularly removed in the aqueous extract and approximately 13–14% in presence of papain.

As it seemed possible that physical changes taking place in the structure of the corn during malting might favour increasing solubility of lipase, a survey was made of changes in extractability during malting. 30-g. samples of germinating barley were dried with acetone, ground and extracted with water. The extracts were then separated from the grists, enzymes which had gone into solution were recovered by precipitation and their activities were determined; residual lipase was estimated in the insoluble material using tributyrin as substrate. Results obtained with the residual grists showed that their activities towards tributyrin paralleled results obtained when triolein was used with unextracted samples taken from the malting floor (see Fig. 2), *i.e.*, there was a decline in activity during steeping and for the first day of flooring, followed by a gradual rise. Extracted lipase declined during steeping and rose again as

flooring progressed: both the initial decline and the final increase were of greater magnitude than was the case with the insoluble enzyme but, nevertheless, the proportion of lipase extractable in water did not at any time exceed 15% of the total.

Preliminary fractionation of lipase.—An aqueous extract was prepared from 1 kg. of barley and ammonium sulphate was added incrementally. Precipitates were collected at 4 levels of concentration of ammonium sulphate and, after they had been redissolved and reprecipitated with acetone, their activities were determined towards tributyrin. Results of these determinations are given in Table III where it can be seen that the most active precipitate was secured with the smallest addition of salt, and that over 80% of the enzyme was displaced from solution by 35% ammonium sulphate. Although the amount of lipase extracted from barley was small in relation to the total enzyme present, nevertheless a considerable enrichment was achieved in terms of enzyme activity in relation to weight of material used as enzyme source.

TABLE III

FRACTIONATION OF CRUDE EXTRACT OF LIPASE

$(NH_4)_2SO_4$ added (%, w/v)	Yield of precipitate (g. per kg. original barley)	Activity* (per 10 mg.)	Total activity	% of soluble lipase in fraction
20	0·51	9·1	464	42
35	0·99	4·6	455	41
50	0·63	2·7	170	15
75 (saturation)	0·17	1·2	20	2

* See text.

DISCUSSION

The results presented above show that barley contains a lipase or lipases, that the activity of the lipolytic material fluctuates during the malting process, and that approximately one-eighth of the lipase can be brought into solution in water. Difficulties of extraction are not uncommon with plant lipases[8] and, even when successful extraction has been achieved (as, *e.g.*, with wheat germ lipase), no information seems to be available about the completeness of extraction from the tissues.

The precipitation characteristics of barley lipase (Table III) suggest that this enzyme may be a protein of very high molecular weight; the extreme heat-lability noted for barley lipase in solution could also be associated with an unusually large molecular size. Size of molecule is unlikely to be the only factor contributing to heat stability, but it may be worth noting that ribonuclease, which is one of the smallest known enzymes, can withstand boiling in acid solution without inactivation. It may further be suggested that extractable wheat germ lipase owes both its extractability and its greater stability to heat to a simple difference in molecular weight of the active component.

Although in solution barley lipase appeared to be extremely heat-sensitive, the precipitate prepared by addition of ammonium sulphate retained full activity for several months. Moreover, in common with many other enzymes, barley lipase showed considerable resistance to heat so long as it was protected within the grain. Thus, in this investigation, an optimum value for lipase activity in ground barley was observed at 48° C., while Maestrini[10] earlier recorded an optimum temperature for insoluble barley lipase at 45° C., with inactivation taking place at 60° C. These figures are in good general agreement with one another, but neither is very meaningful unless notice is taken of the moisture content of the grain. Thus, lipase can be detected in cured malt; hence, the enzyme clearly can survive kilning where temperatures well in excess of 60° C. are reached, but only after the grain is considerably depleted of water.

It is relevant to enquire whether figures derived from the performance of solubilized

lipase have any real meaning in terms of events taking place in the germinating grain. It has been shown (Fig. 1) that there is an optimum level of water for maximum activity of barley lipase in ground grain at 40%, a figure which is not very different from that prevailing during malting. It could thus be argued that determinations made with ground grain give more realistic pictures of events taking place in the living seed than do determinations made with lipase in solution. However, there is no major accumulation of free fatty acids during malting,[9] and it thus seems that the fine grind and precise control of moisture required for demonstration of lipase in barley may serve to allow approximation of enzyme and substrate without simultaneously favouring the action of the mechanism which, in the living corn, further metabolizes the freed fatty acids and prevents their accumulation. It will be appreciated that some degradation of liberated acids within the ground grain may well have taken place during incubation: the results quoted for lipase activity thus represent net action observed in the experimental conditions used.

Considerable uncertainty still exists regarding the reactions involved in fatty acid utilization by plants[12] though, in mammalian tissue, it is now fully established that fatty acids are degraded by β-oxidation and converted to acetyl-coenzyme A derivatives, which then enter the common metabolic pool of the cell. The mammalian enzymes concerned with fatty acid metabolism are all associated with mitochondria, whereas the lipase is a cytoplasmic protein: if similar conditions prevail in plants, then the disruption of cells by grinding may have permitted contact between lipase and triolein, with consequent hydrolysis of the fat, in circumstances where the usual fatty acid degradation has been impaired. Extensive speculation along these lines is at present unwise, but it is as well to recognize that here we have an experimental quandary: solubilized lipase exhibits properties different from those which it manifests in the grain, and use of finely-ground grain allows demonstration of an unknown proportion of the active lipase only because the mechanism which, in life, accomplishes further degradation of the fatty acids has been to some extent impaired.

Of the two substrates used to detect lipase activity in barley, tributyrin proved to be more easily attacked than triolein. This is in agreement with general experience with lipases from other sources, and the question arises whether the same enzyme is responsible for the hydrolysis of both of the esters. On the whole, the balance of opinion seems to be that tributyrinase activity is one aspect of the possible behaviour of a lipase of low specificity (see, *e.g.*, Desnuèlle[2] and Dunkley & Smith[3]), and, although results obtained in the present study do not go far towards solving this problem of specificity, they provide no evidence in favour of distinguishing two separate esterases. Thus, in all experimental work in which both substrates were used, the ratio of fatty acid liberation from tributyrin compared with triolein was the same (9:1) and, in the dissected grain, the proportionate contributions of embryo and endosperm were found to be identical for both substrates. Changes detected in malting also followed parallel courses with the two substrates. In the absence of any evidence to the contrary, therefore, it seems best to accept the simple view that barley contains a lipase of low specificity, and that this enzyme does not alter qualitatively during malting. Certainly the characteristics of the extracted malt enzyme (heat lability, pH optimum, power of attacking tributyrin and triolein) did not differ significantly from those of the barley enzyme. The greater ease of hydrolysis of tributyrin may have a purely physical explanation for, although barley lipase is not readily soluble in water, it is presumably a hydrophilic protein, and a strongly hydrophobic substrate such as triolein could be visualized as being largely out of range of the sphere of influence of the enzyme in presence of excess water; the ester of smaller molecular size might be expected to penetrate more readily to the active centre of the enzyme. The very real problems which are still involved in studying the behaviour of plant lipases in general and barley lipases in particular may thus be essentially biophysical in nature.

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XII

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WATER-SOLUBLE CARBOHYDRATES OF SEEDS OF THE GRAMINEAE

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(With 1 figure in the text)

Summary

Qualitative and quantitative determinations have been made of the sugars, oligosaccharides and water-soluble polysaccharides of the seeds of twenty-two species of the Gramineae.

Although in most species sucrose was the most plentiful free sugar, in *Lolium perenne* and in *Festuca pratensis* a trisaccharide which contained residues of galactose, glucose and fructose and which was chromatographically distinct from raffinose constituted the most abundant oligosaccharide; in *Elymus arenarius* and in *Bromus sterilis* an homologous series of low-molecular fructosans represented the major simple carbohydrate. Similar fructosans were also present in *Agropyron repens*.

Raffinose was present in fifteen of the seeds examined and stachyose in ten of these fifteen raffinose-containing seeds. The contents of raffinose and stachyose were positively correlated.

Water-soluble polysaccharides were obtained in yields approaching 1 per cent of the dry weight of the seeds from certain members of the Bromeae, the Hordeeae, the Festuceae and the Aveneae; yields from members of other tribes were low and predominantly dextrinous in nature.

A pure β-glucosan, resembling that of cultivated barley, has been obtained from Bromus, from Avena and Arrhenatherum, and from Dactylis and other members of the Festuceae. The polysaccharide prepared from Nardus contained a high proportion of mannan and that from Molinia a high proportion of galactan.

When the water-soluble carbohydrates of these seeds are considered in relation to the classification of the Gramineae, it is seen that Bromus is quite distinct from Brachypodium; that the members of the Festuceae here examined show underlying similarity in the composition of their water-soluble polysaccharides, though *Festuca pratensis* and *Lolium perenne* are distinctive in containing an unusual trisaccharide; that Avena and Arrhenatherum differ from the other two members of the Aveneae examined (Holcus and Anthoxanthum) and that the members of the Hordeae constitute a natural group on the basis of their sugars content.

Introduction

Considered as an entity, the Gramineae is a distinctive and well-defined family of flowering plants. However, reasonable unanimity as to the most appropriate allocation within the family of genera to different tribes has not yet been reached, though the present trends in classification involve the creation of a multiplicity of individual tribes.

Thus Haeckel (1887) assigned the British genera of the Gramineae to only 8 different tribes, while Hubbard (1948) refers these genera to 12, Clapham, Tutin and Warburg (1952) to 15 and Hubbard (1954) to no fewer than 19 separate tribes. Thirteen of these 19 tribes each accommodate only one British genus.

As in all other families, the criteria originally selected for arranging individuals in some sort of order are floristic, and consideration of the morphology of the floret and spikelet gives at least a first approximation to the correct determination of the most suitable tribe and genus for any particular species. The rather major rearrangements of the past decade, however, have resulted not from a critical re-examination of details of floral morphology but rather from a broadening of the whole approach to the taxonomy of the grasses. Anatomical features, especially of the leaf, starch-grain structure, and chromosome size and number have provided evidence either for consolidating the position of genera within certain tribes or for evicting others. Brachypodium, for example, which has hovered rather uneasily between the Festuceae and the Hordeeae, has been found to possess chromosomes which are strikingly different from those of either of these tribes; Hubbard (1954) now assigns this genus to a new tribe, the Brachypodieae. In rather a similar manner Nardus, with compound starch grains, is judged to be wholly out of place in the Hordeeae, typical members of which have simple starch grains. This feature, together with certain peculiarities in the epidermal cells, justifies the segregation of Nardus as the single British genus of the tribe Nardeae. Further examples of this type are discussed by Hubbard (1948).

In a consideration of different possible lines of approach to problems of phylogeny, Lawrence (1951) has suggested that many of the findings of plant biochemists have received too little attention from taxonomists. Part, at least, of this neglect may be attributed to the fact that rather few comprehensive comparative studies of the biochemical composition of members of a family, or of related families, have as yet been made, though the overwhelming importance of biochemical considerations in classifying yeasts and bacteria is obvious. Lawrence, however, considers that further biochemical studies of the flowering plants may eventually be of major importance in confirming or rejecting the transfer of genera from one taxon to another. Within the Gramineae, certainly, two general biochemical studies — that of Reichert (1919) dealing with starches and that of de Cugnac (1931) dealing with fructosans — give results which at least partially support Lawrence's contention.

De Cugnac lists a number of genera which he termed 'sacchariferous' since they appeared to contain no sugars or oligosaccharides other than sucrose, glucose and fructose; in particular fructosans were absent. In addition to genera from certain tropical and sub-tropical tribes (e.g. Zea, Saccharum, Oryza, Bambusa) the genera Phragmites, Molinia and Brachypodium, which at the time when de Cugnac prepared his survey were classified in the Festuceae, fall into this group. Phragmites and Molinia are now removed from the Festuceae to the Arundineae and Danthonieae respectively; this separation, originally made on account of differences in spikelet morphology, and subsequently corroborated by cytological and anatomical features, brings Phragmites and Molinia very much nearer to the tropical and sub-tropical groups with which some at least of their biochemical affinities lie. Brachypodium is still linked with Bromus in the Brachypodieae by Clapham *et al.* (1952), though Hubbard (1954) dissociates the two genera into separate tribes. It will be of interest to see whether Brachypodium follows the example of the other two anomalous 'sacchariferous' genera and is eventually aligned with tribes typical of less temperate climates.

De Cugnac's second group of grasses, the 'laevuliferous' species, calls for little comment; this group contains representatives of most of the common tribes of temperate regions, and all members of the group are characterized by the presence of fructosans in the stems or perennating organs. The precise structures of these fructosans are not yet all fully known; all are composed principally of fructose residues and are laevorotatory, with molecular weights of the order of 2500, but some appear to be linear and others cyclic in structure; some contain β-1.2 linked anhydrofructose units while others have 2.6 linkages and yet others contain both types of linkage. More than one type of fructosan may occur in the same species of grass, and though it is possible that related genera may contain structurally similar types, in the absence of fundamental chemical knowledge of fructosan structure, it is wiser to defer consideration of the possible taxonomic implications of the distribution of these assorted compounds. Review articles devoted to fructosans (Archbold, 1940; Whistler and Smart, 1953) fully illustrate the range and complexity of these carbohydrates.

Most of de Cugnac's work relates to the vegetative parts of grasses and surprisingly little is known about the carbohydrate content of grass seeds, apart from their starch. However, a general survey of the water-soluble carbohydrates of common cereal grains (MacLeod and Preece, 1954) has shown that considerable differences are possible both in the relative quantities present and in the chemical constitution of certain carbohydrate fractions. It seemed of interest, therefore, to extend this work from the cereals to include a representative series of seeds of wild grasses, so as to establish the range of composition possible within a complete natural family; even if the results of such a survey shed no light on taxonomic problems, they should be at least of some general biochemical interest.

It must be emphasized at the outset that very great caution is required in interpreting the results of a survey such as this. Wild grasses do not lend themselves to cultivation under controlled experimental conditions and there is very clear evidence to show that, for example, the available mineral nutrients in the soil may profoundly influence the total quantities of materials synthesized and stored as reserves. In general these nutritional differences influence the total quantities of materials synthesized rather than the nature of the products formed, but weather conditions, for example, may be responsible for suppressing or enhancing the formation of certain reserve carbohydrates in the leaves (see, e.g. Yemm, 1935). It seems clear, however, that these environmental differences exert a very much smaller influence on the carbohydrates of the seed than they do on the leaf carbohydrates; thus Archbold (1938) found that under conditions of nitrogen-deficiency there was a ten-fold increase in the amount of fructosan stored in the stem, as compared with only 11 per cent increase in the ears. Nevertheless, in considering the results presented below, the possible effects of cultural and climatic differences operating throughout the life cycle of the plant and finally finding expression in the carbohydrate balance of the ripe grain, must not be overlooked.

EXPERIMENTAL

Materials — Grass 'seeds' which are available commercially were purchased locally; other material was collected mainly from southern Scotland, and the seeds were cleaned from extraneous matter and air-dried in the laboratory. Collections were made only from plants which had begun to shed their seeds so that all samples were, as far as could be judged, uniformly ripe. With the exception of Spartina, in which no true resting

period occurs, really ripe grass seeds undergo no major changes until they are moistened and in the dried seeds no deterioration appeared to take place during the few months of storage which was inevitable before analysis. The 'seeds' of all grasses are not equivalent structures and it is quite impossible to dissect out sufficient caryopses or to include the appropriate amounts of glumes, etc., to render all the samples morphologically similar; however, previous work has shown that, for barley at least, sugars (MacLeod, 1952) and water-soluble polysaccharides (Preece and Mackenzie, 1952) are of very minor importance in the husk. A rapid examination of the aqueous extracts of the glumes of certain wild grasses indicated that this was true of these species also, and it is assumed that no great extra contribution of carbohydrate is made from the integuments. The presence or absence of glumes, lemmas and paleas, with or without awns, affects the comparative dry-weight results and, for many purposes, results are more usefully considered as quantities of carbohydrate per seed or, more realistically, perhaps, per million seeds. The origins of the samples, the dry weight of 1000 seeds, as analysed, and the proportion of husk present when that could be determined are given in Table 1.

Table 1. *Materials used*

Tribe*	Genus and species*	Dry weight of 1000 seeds (as analysed)	Origin of sample
Bromeae	*Bromus sterilis* L.	8.29 g. (20% husk)	Waste ground, Edinburgh
Brachypodieae	*Brachypodium sylvaticum* (Huds) Beauv.	4.12 g. (25% husk)	Woodland, Bridge of Allen
Hordeeae	*Agropyron repens* (L.) Beauv.	3.59 g. (30% husk)	Waste ground, Edinburgh
	Elymus arenarius L.	20.40 g. (26% husk)	Sand dunes, Longniddry
Glycerieae	*Glyceria plicata* Fries	0.86 g. —	Pond West, Edinburgh
Festuceae	*Festuca pratensis* Huds.	2.24 g. —	Commercial
	Lolium perenne L.	1.99 g. (19% husk)	Commercial
	Poa trivialis L.	0.15 g. —	Commercial
	Dactylis glomerata L.	0.73 g. —	Commercial
	Cynosurus cristatus L.	0.54 g. —	Commercial
Aveneae	*Arrhenatherum elatius* (L.) J. and C. Presl	2.56 g. (30% husk)	Waste ground, Edinburgh
	Avena fatus L.	24.60 g. (48% husk)	Arable land, Rothamsted
	Holcus lanatus L.	0.34 g. (shelled)	Commercial
	Anthoxanthum odoratum L.	0.53 g. (40% husk)	Wood and heath, Bridge of Allen
Phalarideae	*Phalaris canariensis* L.	6.80 g. —	Commercial
Agrostideae	*Ammophila arenaria* L.	1.70 g. —	Sand dunes, Gullane
	Agrostis canina L.	0.06 g. —	Commercial
	Phleum pratense L.	0.41 g. —	Commercial
Nardeae	*Nardus stricta* L.	0.68 g. —	Various moors, East Scotland
Danthonieae	*Molinia caerulea* (L.) Moench	0.35 g. —	Damp moorland, East and West Scotland
	Sieglingia decumbens (L.) Bernh.	2.34 g. —	Heath, Fort William
Spartineae	*Spartina townsendii*	10.02 g. (41% husk)	Southampton Water

* Nomenclature and classification as in Hubbard (1954).

Methods

Water-soluble carbohydrates were extracted as two fractions, the alcohol-soluble fraction which includes sugars and oligosaccharides and the alcohol-insoluble fraction which can be subsequently removed with water and which includes polysaccharides resembling the cereal gums (Preece and Mackenzie, 1952).

Sugars and oligosaccharides

A sample of dry seeds (*ca.* 50 g. for a pilot investigation to determine the nature and approximate quantities of the selected carbohydrate fractions, and either 100 or 200 g.

for a full-scale study) was ground and exhaustively extracted with boiling 80 per cent ethanol. This treatment inactivated enzymes and brought into solution all sugars and oligosaccharides, including certain of the fructosans. The residue was air-dried and reserved, the ethanolic extract was filtered bright and the ethanol removed by distillation. After storage overnight in the refrigerator when some fatty material was deposited, the aqueous extract was filtered bright and made to a known volume. An aliquot was used for quantitative determination of reducing sugars and the remainder was concentrated and examined chromatographically. Butanol-acetic acid-water proved the most useful solvent system for sugar separations. Individual sugars and oligosaccharides were eluted from the appropriate regions of chromatograms and the relative proportions of the different eluates were determined, after hydrolysis if necessary, by the Somogyi (1945) technique. The total amounts of the individual sugars present in the original sample could then be calculated (see MacLeod, 1952, for a fuller account of this method). This general procedure sufficed for quantitative determination of the chromatographically separable sugars and oligosaccharides without necessarily providing full identification of the different compounds concerned, and special methods were required to identify two of the oligosaccharides encountered. These methods are described below.

Water-soluble polysaccharides

The dried sugar-free residue was extracted for 3 half-hour periods with water at 40° C. After filtering the combined extracts and washings water-bright through kieselguhr and concentrating to about 200 ml., an equal volume of mixed Fehling's solution was added, followed by acetone to 40 per cent of the total volume. The precipitate was dissolved in 2N HCl, reprecipitated by acetone, and taken to dryness. This procedure does not lead to complete recovery of the soluble polysaccharide (Hobkirk (1955) quotes recovery figures for barley extracts treated in this manner of 80 per cent of the colloidal carbohydrate) but the preparation of the polysaccharide via a copper complex does give a product which is virtually protein-free and ash-free.

The sugar units constituting the polysaccharide were identified by hydrolysing a 20 mg. sample with N H_2SO_4 and separating the neutralized salt-free hydrolysate chromatographically. Quantitative estimation of the different sugar units present was carried out on eluates from chromatograms, in a manner similar to that used for the free sugars, but with butanol-ethanol-water as solvent system. When the yield of polysaccharide was sufficiently large, fractionation by means of ammonium sulphate was carried out. In the hands of Preece and Mackenzie (1952) this technique has proved outstandingly useful for separating pure β-glucosan from crude water-soluble polysaccharides of barley and oats, and, since a survey of cereal grains (MacLeod and Preece, 1954) had shown a remarkable variation in the content of this particular carbohydrate, it was felt that the presence or absence of this β-glucosan might prove a point of some significance.

The fractions obtained by ammonium sulphate treatment were hydrolysed and examined chromatographically, and where possible determinations of specific rotation and of viscosity were carried out.

RESULTS

Sugars, oligosaccharides and fructosans

As can be seen from Table 2, the range of sugars and oligosaccharides encountered in the samples of grass seeds examined included glucose and fructose, sucrose, raffinose and

a second trisaccharide which was chromatographically distinct from raffinose, a tetrasaccharide resembling stachyose, and a series of low-molecular fuctosans. Trace amounts of galactose and arabinose, too small to be estimated with accuracy, were also occasionally present. Glucose, fructose and sucrose occurred in all the seeds, though the monosaccharides were usually rather minor components. With four exceptions (Bromus, Elymus, Festuca and Lolium) sucrose was quantitatively the most important simple carbohydrate present. In Bromus and Elymus the secondary importance of sucrose was due to the accumulation (or persistence) of a high concentration of fructosans, and in Festuca and Lolium an unfamiliar trisaccharide represented the major sugar present in

Table 2. *Sugars and oligosaccharides of grass seeds*

Genus (species as in Table 1)	mg. sugar per 100 g. dry weight of seeds (glucose equivalent, after hydrolysis where necessary)					
	Glucose	Fructose	Sucrose	Trisaccharide	Stachyose	Fructosan
Bromus	38	120	735	—	—	2211
Brachypodium	150	183	1380	286	220	—
Agropyron	158	170	194	72	—	274
Elymus	50	83	570	122	—	817
Glyceria	205	189	676	110	—	—
Festuca	49	67	168	324*	—	—
Lolium	147	127	472	970*	—	—
Poa	72	101	1010	130	64	—
Dactylis	55	37	600	184	75	—
Cynosurus	105	85	815	200	48	—
Arrhenatherum	220	200	375	Trace	±	—
Avena	106	94	626	170	134	—
Holcus	55	40	340	315	185	—
Anthoxanthum	86	88	245	154	19	—
Phalaris	19	26	286	81	—	—
Ammophila	280	270	885	330	Trace	—
Agrostis	31	32	417	133	78	—
Phleum	54	28	528	70	24	—
Nardus	198	152	978	217	—	—
Molinia	375	563	920	290	—	—
Sieglingia	69	75	1085	164	105	—
Spartina	344	286	10700	—	—	—

* Raffinose isomer: other trisaccharide entries are authentic raffinose.

the ripe seed. This trisaccharide was obtained in solution free from other carbohydrates by elution of the complete sugar mixture with increasing concentrations of ethanol from a charcoal-celite column (Whistler and Durso, 1950) when a pure sample was obtained in the 15 per cent ethanol fraction. Although this trisaccharide was distinctly more mobile than raffinose in butanol-acetic acid-water, it agreed with raffinose in yielding equal quantities of galactose, glucose and fructose after hydrolysis with N H_2SO_4 and in hydrolysing to fructose and a reducing disaccharide in the presence of invertase at pH 4.6. Results of emulsin treatment were somewhat equivocal and pending its fuller characterization this trisaccharide is referred to as the raffinose isomer; it is of interest to note that this trisaccharide appeared to be readily mobilized when Lolium seeds germinated, thus recalling the behaviour of raffinose in germinating barley. Raffinose itself could not be detected in Lolium and Festuca.

The tetrasaccharide was obtained only from seeds which contained raffinose, though not all raffinose-containing seeds also yielded the tetrasaccharide. Chromatographically this oligosaccharide had the same mobility as stachyose in several solvent systems, and treatment with acid or with invertase gave the products which would be expected from stachyose. Ten of the seventeen seeds which contained raffinose also contained measurable amounts of the presumed stachyose, and as raffinose content increased, stachyose

content increased also (Fig. 1). The correlation between raffinose content and stachyose content is r = +0.82 which, with ten results available, is significant at the level. It is of interest to note that in Festuca and Lolium, where the raffinose isomer formed the major free sugar of the grain, no tetrasaccharide could be detected.

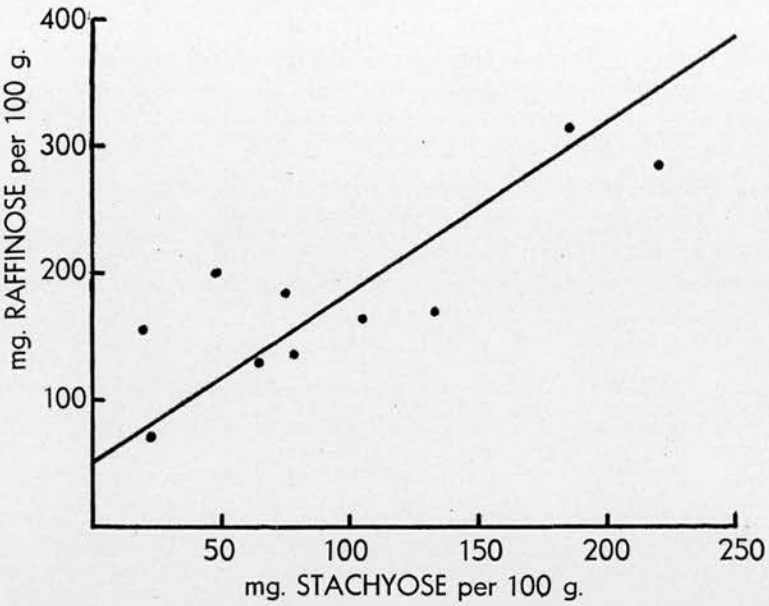

Fig. 1. Raffinose and stachyose in the seeds of ten species of grasses. Stachyose = 0.75, raffinose −40.

On the basis of the nature of the sugars and oligosaccharides contained in their seeds, and ignoring quantitative differences in the amounts of the individual sugars present, these twenty-two species of grass may be arranged in six different groups (Table 3).

Table 3. *Grass seeds grouped according to sugars content*

	Sugars present	Genera in group (species as in Table 1)
Group 1	Hexoses and sucrose only	*Spartina*
Group 2	Hexoses, sucrose and homologous series of fructosans	*Bromus*
Group 3	Hexoses, sucrose, homologous series of fructosans and raffinose	*Elymus, Agropyron*
Group 4	Hexoses, sucrose and raffinose	*Glyceria* *Phalaris* *Nardus* *Molinia*
Group 5	Hexoses, sucrose, raffinose and stachyose	*Brachypodium* *Poa, Dactylis, Cynosurus* *Arrhenatherum, Avena, Holcus, Anthoxanthum* *Ammophila, Agrostis, Phleum*
Group 6	Hexoses, sucrose and isomer of raffinose	*Festuca, Lolium*

Water-soluble polysaccharides

Yields of the crude products ranged from 0.11 per cent to 1.9 per cent of the dry weight of the seeds, the physical nature varied from pulverulent and forming a watery solution to fibrous and forming a viscous aqueous solution, and the specific rotation ranged from +135° to −35°; clearly, then, although the principal hydrolysis products

were in almost all cases restricted to glucose, xylose and arabinose, several different materials were represented in this series of carbohydrates. Details of yields, physical characteristics and proportions of constituent sugar units of the products (all of which were virtually ash-free and protein-free) are given in Table 4.

Table 4. *Yields, physical characteristics and constituent sugar units of water-soluble polysaccharides*

Genus (species as in Table 1)	Yield (mg. per 100 g.)	α_D	Specific viscosity	Sugars after hydrolysis (%, glucose equivalent) G†	X†	A†	Other
Bromus	1890 (Fibrous)	− 35	0.66	57	25	18	—
Brachypodium	1130	0	0.69	52	17	31	—
Agropyron	780	+ 85	0.17	81	9	10	—
Elymus	390 (Fibrous)	+ 25	0.70	56	23	21	*
Glyceria	190	+105	0.11	72	3	25	—
Festuca	950 (Fibrous)	+ 15	0.80	78	9	13	—
Lolium	850	+ 90	0.29	88	7	5	—
Poa	280	+ 31	0.18	62	9	29	—
Dactylis	1140 (Fibrous)	− 10	0.65	89	2	9	—
Cynosurus	800	+ 50	0.27	84	7	9	—
Arrhenatherum	590 (Fibrous)	0	0.43	85	4	11	*
Avena	1030 (Fibrous)	+ 10	1.26	90	4	6	—
Holcus	180	—	—	90	4	6	—
Anthoxanthum	360	+135	0.05	88	2	10	—
Phalaris	170	+110	0.15	92	2	6	—
Ammophila	160	—	—	72	12	16	—
Agrostis	230	+ 97	—	78	7	15	
Phleum	280	+ 70	0.13	85	7	8	—
Nardus	700	—	—	75	1	5	Mannose 19
Molinia	180	+ 35	0.10	50	6	21	Galactose 23
Sieglingia	110	—	—	73	6	21	—
Spartina	680	+100	0.15	93	1	6	—

Preparations not marked fibrous were pulverulent.
* Trace quantities of galactose present.
† G = Glucose; X = Xylose; A = Arabinose.

Fractionation by means of ammonium sulphate gave results wholly in accord with previous experience of this procedure; β-glucosan of high viscosity was precipitated at 20-30 per cent saturation, substantially free from pentosan, arabo-xylan was precipitated at 50 per cent saturation, contaminated with α-glucosan, and a mixture of dextrinous material and pentosan could be recovered from the mother liquor. Results of fractionation of products obtained in reasonable yields are given in Table 5; it should be noted

Table 5. *Ammonium sulphate fraction of polysaccharides*

Origin of polysaccharide (species as in Table 1)	Percentage of total recovery at saturation of 30% $(NH_4)_2SO_4$	50% $(HN_4)_2SO_4$	Mother liquor
Bromus	56 (−10°)	34	10
Agropyron	0	65	35
Elymus	0	80	20
Festuca	58 (−9°)	17	25
Lolium	14	64	12
Dactylis	84 (−8°)	0	16
Cynosurus	10	40	50
Arrhenatherum	56 (−9°)	26	18
Avena	77 (−8°)	13	10

Fractions precipitating with 30 per cent $(NH_4)_2SO_4$ were virtually pure glucosan. Figures in parentheses represent specific rotations of fractions.

that the figures quoted represent percentages of the total recovery after ammonium sulphate fractionation and since it cannot be assumed that losses from all fractions are proportional, these figures do not accurately indicate the original compositions of the crude polysaccharides.

The water-soluble polysaccharides from Nardus and Molinia differed from preparations from all other grass seeds in containing significant amounts of, respectively, mannan and galactan. Fractionation of these atypical polysaccharide mixtures has not yet proved possible owing to the difficulty of collecting sufficient quantities of ripe seeds of the two grasses, but a further study of the NaOH-soluble hemicellulose from Nardus indicated that the mannan was restricted to the water-soluble material. Classification of the seeds with respect to the nature of the water-soluble polysaccharides present is shown in Table 6.

Table 6. *Grass seeds grouped according to nature of water-soluble polysaccharides present*

	Distinguishing characteristic	Genera in group (species as in Table 1)
Group 1	β-glucosan present	*Bromus*
		Arrhenatherum, Avena
		Dactylis, Festuca, Poa, Lolium**
		*Cynosurus**
Group 2	Mannan approximately 20 per cent of polysaccharide	*Nardus*
Group 3	Galactan more than 20 per cent of polysaccharide	*Molinia*
Group 4	Pentosan more than 20 per cent of polysaccharide	*Brachypodium*
		Agropyron, Elymus
		Glyceria
		Sieglingia
Group 5	α-glucosan more than 80 per cent of polysaccharide	*Phalaris*
		Holcus, Anthoxanthum
		Ammophila, Agrostis, Phleum
		Spartina

* Minor constituent of crude polysaccharide.

No obvious relationship could be detected between the size of the grains, with or without husk and their contents of sugars or water-soluble polysaccharides. (Tables 1, 2 and 4.)

DISCUSSION

In attempting to assess the significance of the results presented above, two apparently distinct questions suggest themselves, viz. (*a*) what light do these observations shed on the origins, metabolic significance, and inter-relationships of the different carbohydrates investigated? and (*b*) to what extent is the carbohydrate balance of the ripe seed typical of a genus, tribe, or other taxon as normally distinguished by other, more familiar morphological criteria? These two problems may not be as distinct as appears at first sight. If the deposition and utilization of carbohydrate in the developing embryo and endosperm is visualized as proceeding along a number of individual lines, then the point reached on any given line at ripeness will depend on the total amounts of raw material available for elaboration into that particular carbohydrate, on the activities of the enzymes concerned in catalysing its synthesis, and on the presence and activities of hydrolytic and glycolytic enzymes capable of accomplishing the degradation of the deposited material. Presumably the enzyme balance is, fundamentally, gene-controlled, so that the amounts of the various carbohydrate fractions present at ripeness must depend on the genetic constitution of the plant. Whether the carbohydrate status of the seed is as constant a characteristic as, for example, the hairy appendage on the ovary of Bromus and

Brachypodium is a question whose answer must await the amassing of fuller analytical data. However, despite the postulated relationship between the biochemical aspects of the problem and their possible taxonomic implications, it is simpler in the first instance to discuss these two facets separately.

Biochemical aspects

The predominance of sucrose amongst the free sugars and the relative insignificance of the monosaccharides is not unexpected, but the frequency with which raffinose and stachyose occur in this series of seeds is noteworthy, and directs attention to the as yet unsolved problem of the origin of raffinose in storage organs and the mechanism concerned in its mobilization when growth is resumed. Stachyose and raffinose can usefully be considered together for, although little information is available about the mode of formation of tetrasaccharides, it is not impossible that stachyose may be derived from raffinose by addition of a terminal galactose residue; such formation can be induced in barley embryos (which do not normally contain stachyose) by infiltration of a concentrated solution of raffinose and subsequent exposure to oxygen. A consideration of the regression equation for the line plotted in Fig. 1 ($S = 0.75$, $R - 40$) suggests that tetrasaccharide deposition may be initiated when a certain minimal level of trisaccharide is attained and that, once this threshold value has been passed, approximately equal quantities of the two ligosaccharides accumulate in the seeds. Clearly, however, a high concentration of raffinose is not the only prerequisite for stachyose accumulation in the seed as in many species, notably Molinia, Nardus and cultivated barley and rye, high concentrations of raffinose are not accompanied by any significant amounts of stachyose. Indeed, in Molinia the 'extra' galactose units appear as a water-soluble galactan.

It will be recalled that Colin and Belval (1934) have shown that raffinose content tends to diminish in ripening oat seeds until, at harvest, this sugar may not be detectable; in oats, therefore, the concentrations of the sugar present at 'ripeness' represent the residue left after some of the material initially present has been utilized during ripening. The work of Archbold (1938) has established the fact that the fructosans of ripe barley may also represent a residue in the grain — though these same materials appear to be a climax of carbohydrate accumulation in the internodes. The starch content of barley at full ripeness, on the other hand, is the climax of a synthesis carried out mainly by the developing ears themselves and the immediately adjacent leaves (Porter *et al.*, 1950). With barley again, the raffinose content per 1000 corns appears to fall slightly during the early stages of ripening of the grain, and thereafter to remain constant during the last few weeks of ripening (Harris and MacWilliam, 1954); conditions conducive to raffinose utilization would thus appear to be present in the immature grain and again in the germinating seedling (MacLeod, 1957), but absent from the nearly ripe grain.

In different species, different points of balance for different sugars and oligosaccharides therefore appear to be attained at ripeness. Thus, fructosans are amply present in Bromus, Elymus and Agropyron, whereas in Avena a similar series of fructosans present in the immature seeds, is virtually undetectable at ripeness. Similarly raffinose, which diminishes in quantity throughout the entire ripening process of oats, reaches a point of equilibrium in barley, only to disappear with startling suddenness when seedling growth begins. In this connection it is of interest to note that certain enzyme systems may show developmental behaviour similar to that postulated above for the sugars; thus, in five different cereal grains (barley, wheat, rye, oats and maize) the different levels of

activity of three enzymes involved in degrading β-glucosan to glucose is most marked (Preece, Aitken and Dick, 1954). If this concept of different levels of different carbohydrates present at maturity, and mediated by different enzymic activities within the seeds, is a valid one, then the overall pattern of carbohydrate dispositions at ripeness may well be unique to a given species, or group of species, though the total quantity of any fraction present may be expected to vary within rather wide limits, according to climatic and growth conditions acting on the developing plant. Such a concept, however, does not help to explain the presence in two species (Lolium and Festuca) of a trisaccharide apparently distinct from any other found in grass seeds; the presence of an enzyme system peculiar to these two grasses must be postulated.

Though similar conditions may apply to the water-soluble polysaccharides as to the simpler sugars, so little information is available about their deposition and utilization in plants in general that any speculation about their origins and functions in grass seeds must inevitably be limited. However, with the exception of the preparations from Bromus, the water-soluble polysaccharides of the seeds of wild grasses proved much less interesting than comparable preparations from cultivated cereals. Laevorotatory glucosan of high viscosity was the major constituent of the polysaccharide from only five species of seed, and represented a minor constituent of three others; this material had a specific rotation of approximately $-9°$ and showed precipitation characteristics similar to those of the β-glucosan isolated from barley by Preece and Mackenzie (1952). Barley β-glucosan contains approximately equal numbers of β-1.3 and β-1.4 linkages (Aspinall and Telfer, 1954) thus to some extent resembling lichenin, a glucosan which contains 30 per cent of β-1.3 and 70 per cent of β-1.4 linkages. Barley β-glucosan is eliminated during malting, by the joint action of a disaggregating endo-β-glucosanase, an exo-β-glucosanase, and cellobiase and the sugars resulting from hydrolysis of the polysaccharide are presumably incorporated in the common metabolic pool for re-utilization in respiration and synthesis. In addition to this soluble β-glucosan, certain grass seeds, notably those of barley and of Bromus spp. (MacLeod, McCorquodale and Rennie, unpublished) also contain water-insoluble β-glucosan which can, however, be extracted with cold 4 per cent NaOH, and which is therefore classed as a typical hemicellulose. When barley germinates, this hemicellulose glucosan is solubilized and thereafter degraded in a manner similar to that which operates with the initially soluble β-glucosan and it must therefore be regarded as a reserve of polysaccharide utilizable by the developing seedling. The structure of the soluble β-glucosan, its behaviour during germination, its relationship to the insoluble hemicellulosic glucosan and the characteristics of certain of the enzymes which catalyse its degradation thus seem to be reasonably well understood, though there is at present no information available about the mechanism governing its initial synthesis by the parent plant.

The results of the present preliminary investigation suggest that there is a small group of wild grass seeds which resemble barley in containing β-glucosanic material, though in the absence of detailed structural studies it cannot be stated definitely that precisely the same material is involved.

The water-soluble pentosans, which were obtained in reasonable yields only from Bromus, Agropyron and Elymus, belong to a group of polysaccharides about whose metabolism remarkably little is known. Many pentosanic hemicelluloses have been subjected to structural investigation, but these hemicelluloses have traditionally been regarded as exclusively cell-wall constituents, which, after incorporation by means unknown into the cell wall, have remained out with the ordinary metabolic activities of

the cell. However, since the days of Brown and Morris (1890), brewing biochemists have shown considerable interest in the transformations of pentosans in the endosperm of barley, as the 'modification' of the grain during malting at least partially depends on solubilization of the constituents of the cell walls. The general picture of behaviour in barley is complicated by the synthesis of pentosan in the seedling concurrently with degradation of similar material in the endosperm, but it is clear that considerable transformations of pentosam are accomplished during germination (see Preece, 1954 for a summary of this work). It may well be that in certain grasses the pentosan of the endosperm forms a reserve which may make a significant contribution to seedling development. Certainly in *Bromus sterilis*, where the high content of water-soluble pentosan is a reflection of very large amounts of true hemicellulose, the thick cell walls of the endosperm vanish with spectacular suddenness at about the fifth day of germination; studies of carbohydrate transformations in germinating Bromus, at present in progress, should provide some information about the possible utilization of pentosan fragments.

The pulverulent soluble α-glucosan with a high positive specific rotation has not been studied in detail in the present investigation. Never present in high yield, it gave a strong blue colour with iodine and appeared to resemble starch. The recently described phytoglycogen from maize (Peat *et al.*, 1956) in some respects resembles these α-glucosan fractions, but none of the seeds examined gave sufficiently high yields to render further investigation possible.

Two grass seeds yielded water-soluble polysaccharides containing significant quantities of sugar units other than glucose, xylose and arabinose. These were Molinia, where approximately one-quarter of the crude polysaccharide was formed of galactose units, and Nardus, where 19 per cent of mannose was detected in the hydrolysate. In their exhaustive fractionation of the water-soluble polysaccharides of barley, Preece and Mackenzie (1952) were able to detect mannose and galactose in the hydrolysates of some of the more soluble fractions, but in very small amounts. On a dry weight basis the content of galactan in the sample of Molinia here examined was approximately 25 times as great as that of barley, and the content of mannan in Nardus was of the order of 100 times as great as that of barley. It may be argued that, if sufficiently large quantities of starting materials are taken, then all plant materials may be shown to contain all plant sugars and polysaccharides, although occasionally in infinitesimal quantities, but the strikingly different balance of the units present is noteworthy.

One final point of biochemical interest may be mentioned. It was recorded earlier (MacLeod and Preece, 1954) that, in the seeds of cultivated cereals, species with a high pentosan content also showed a high content of the homologous series of water-soluble fructosans. This rather surprising parallel, for which no obvious explanation suggests itself, appears to extend to the wild grasses, where the fructosan-rich species (Bromus, Agropyron, Elymus) are also those with a concentration of water-soluble pentosan.

Taxonomic implications

If Table 1 and Table 3 are considered in conjunction, it will be seen that two tribes of the Gramineae (as represented by the members studied here) are uniquely characterized by the sugar contents of their seeds. These are the Bromeae, containing fructosans but lacking raffinose, and the Hordeeae, containing both fructosans and raffinose. Although results for only one member of the Bromeae are quoted here, it may be mentioned that in addition to *Bromus sterilis* (*Anisantha sterilis* (L. Nevski)), three other species of Bromus

were examined, viz. *B. asper* Murr. (*Zerna ramosa* (Huds.) Lind.), *B. mollis* agg. and *B. erectus* Huds. (*Zerna erecta* (Huds.) Panz.). All four species gave similar results in respect of both sugars and water-soluble polysaccharides, so clearly the four different species of Bromus or, if the sub-division of the genus Bromus is preferred, four species from three related genera, may be readily distinguished from all other grasses on the carbohydrate composition of their seeds. Again, although only two members of the Hordeeae are discussed here, it will be recalled that the cultivated members of this tribe (wheat, barley and rye) show a pattern of sugar distribution exactly similar to that recorded for the two wild genera, Elymus and Agropyron.

Spartina, with no sugars more complex than sucrose, might also be considered to be fully characterized by its sugar content, but the absence of a true resting stage in the seeds of this grass makes any statement on the sugar content of the 'seeds' of doubtful value as, in certain instances at least, the more complex oligosaccharides of the cereal seeds vanish in the early stages of seedling growth before any gross morphological change is detectable.

All members of the Aveneae and Agrostideae examined exhibited the commonest pattern of sugar distribution (hexose, sucrose, raffinose and stachyose) as did Brachypodium and three of the Festuceae (Poa, Dactylis and Cynosurus). Clearly, in its content of free sugars and also in the nature of the water-soluble polysaccharides present (Table 2) Brachypodium is quite distinct from Bromus, though Clapham *et al.* (1952) assign both to the tribe Brachypodieae.

Festuceae is the only tribe whose members are distributed between two groups on the basis of their contents of sugars; Festuca and Lolium are again uniquely characterized by their possession of a peculiar trisaccharide, and the close relationship between these two genera is further attested by the occurrence of the hybrid Festucalolium (*F. pratensis* × *L. perenne*). In the past the Festuceae harboured many genera which have now been evicted from the tribe (e.g. Phragmites, Sieglingia, Molinia, Bromus, Brachypodium and Glyceria) and these refugee members have now (Hubbard, 1954) been assigned to different, isolated tribes. Hubbard (1948) regards the Festuceae as the most ancient tribe of the group of mainly temperate-climate grasses, and it may well be that, despite the eviction of some of the obviously less desirable members, the residue is still rather heterogeneous with regard to both biochemistry and floral morphology.

The species comprising Group 4 of Table 3 (characterized by the apparent absence of oligosaccharides more complex than raffinose) belong to four separate rather isolated tribes, three of which (Nardeae, Danthonieae and Glycereae) are not clearly related to other well-authenticated tribes. Except in so far as it confirms the fact that Nardus does not show any close affinities to the Hordeeae, the sugar content of the seeds offers no useful clues to the possible relationships of these tribes.

With regard to the water-soluble polysaccharides (Table 6), it is clear that Nardus (Nardeae) and Molinia (Danthonieae) are uniquely characterized by their possession of, respectively, very high concentrations of mannan and galactan. According to Hubbard (1948), 'Nardus presents a unique combination of characters, suggesting that it is a relict of a very ancient group of grasses from which both festucoid and panicoid groups of tribes have been derived.' While Nardus shares its pattern of sugar distribution with tribes typical of the panicoid group (Maydeae as represented by cultivated maize, and Paniceae as represented by *Setaria italica*) and with one tribe typical of the festucoid group (Phalarideae) the remarkable mannan content of the polysaccharide is not shared by any other tribe. Molinia is tentatively assigned to the Danthonieae by Hubbard

(1948) along with Sieglingia, which it resembles in certain morphological features of the leaf, and in basic chromosome number; it differs from Sieglingia in the rather high content of galactan, though it resembles it in the general pattern of sugar distribution. Certainly the water-soluble carbohydrates of Molinia show no relationship of this genus with the Festuceae, with which it was formerly classed.

The grasses whose seeds are rich in β-glucosan belong to the Bromeae, the Festuceae, or the Aveneae. The Bromeae have already been shown to constitute a very distinctive group in virtue of their content of sugars and oligosaccharides. In the Festuceae, Dactylis and Festuca contain large amounts of β-glucosan, while Cynosurus, Poa and Lolium contain very much smaller quantities of this material. Only two of the Aveneae (Avena and Arrhenatherum) contained typical β-glucosan; it is of interest to note that the two genera which lack β-glucosan have somewhat tenuous affinities with this tribe. Thus Hubbard (1948) states that Holcus has no obvious near relatives in the Aveneae, while Clapham *et al.* (1952) assign Anthoxanthum to the Phalarideae.

The seeds with pentosan-rich polysaccharides all belong to the Bromeae or to the Hordeeae. The cultivated cereals from the Hordeeae (barley, wheat and rye) also contain relatively high amounts of water-soluble pentosan within their seeds, but only barley is rich in β-glucosan. The production of artificial hybrids (Agropyron × Triticum; Agropyron × Elymus indicates the close natural relationship between certain members of the tribe and the occurrence of a putative natural hybrid of Hordeum and Elymus (Hordelymus) together with the distinctive grouping of spikelets of the two genera has been considered firm evidence for the close relationship of Hordeum and Elymus. On the basis of their content of water-soluble polysaccharides, it would appear that Elymus and Agropyron have rather strong affinities with rye and wheat, while barley occupies a more isolated position; from the point of view of sugars content, on the other hand, the tribe appears to be a homogeneous one.

In summarizing the taxonomic implications of this study of the water-soluble carbohydrates of seeds of the Gramineae, the following points may be considered of particular significance:

1. Nardus occupies an isolated position, in virtue of its high content of water-soluble mannan.

2. The Bromeae form a very natural tribe, quite distinct from the Brachypodieae, the Festuceae and the Hordeeae.

3. The Hordeeae form a natural tribe, as revealed by their sugars content; on the basis of their content of soluble polysaccharides, Hordeum itself is rather distinct from the other genera examined.

4. All the Festuceae contain soluble β-glucosan in greater or lesser amounts, but two genera (Lolium and Festuca) are distinctive in containing an unusual trisaccharide.

5. Two of the Aveneae, as recognized by Hubbard (1948) differ from the other two (Avena and Arrhenatherum) in completely lacking β-glucosan. The inclusion of these two genera, Anthoxanthym and Holcus, in the Aveneae, is slightly suspect on morphological grounds; in their contents of soluble carbohydrates, both show affinities with the Agrostideae and, to a lesser extent, with Phalaris.

In conclusion, it must be stressed that the findings presented here represent the results of what is essentially a preliminary study. The figures quoted were broadly reproducible for separate collections of a species in different seasons and from different sources and, with Bromus and Avena, different species of the two genera gave essentially similar analyses, but, in the absence of more comprehensive data it must not be lightly assumed

that all species of, e.g. Festuca will inevitably contain water-soluble carbohydrates identical with those of *F. pratensis*.

Nevertheless it is interesting to note that, using the criteria outlined above and ignoring all morphological data, over half of the species examined could be distinguished absolutely by their contents of water-soluble carbohydrates. This material normally represents rather less than 5 per cent of the dry weight of the seeds, and it may be suggested that a detailed study of other components might yield results of far-reaching taxonomic importance.

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Trisaccharides of *Lolium* and *Festuca*

In the course of an investigation into the distribution of water-soluble carbohydrates in seeds of the Gramineae[1], it was observed that two species of grass (*Lolium perenne* L. and *Festuca pratensis* Huds.) contained in their caryopses substantial amounts of a trisaccharide which was not detected in any of the thirty other wild grasses and cereals examined. This non-reducing trisaccharide (Fig. 1, *X*) had a specific rotation of +90·5° (without recrystallization), yielded equal amounts of galactose, glucose and fructose on acid hydrolysis and was readily broken down to fructose and a reducing disaccharide in the presence of invertase or very dilute acid. This ease of hydrolysis suggests that the trisaccharide resembles raffinose in containing a terminal β-fructofuranoside residue. Iodine oxidation[2] indicated that the galactose residue was at the non-reducing end of the disaccharide, and electrophoretic mobility of the disaccharide in borate was intermediate between the mobilities of laminaribiose and melibiose. Movement in borate is allied to the mode of linkage of sugar residues[3], disaccharides containing 1,2 or 1,4 linkages being of much lower mobility than those containing 1,3 or 1,6 linkages. The disaccharide under consideration would therefore appear to contain either a 1,3 or a 1,6 linkage. Although the electrophoretic behaviour in borate does not distinguish between α and β linkages, the high positive rotation of the trisaccharide suggests that the galactose and glucose residues are α-linked, and, since 6-glucose-α-galactoside is melibiose, it seems probable that the newly isolated disaccharide is 3-glucose-α-galactoside. The trisaccharide from *Lolium perenne* and *Festuca pratensis* is therefore tentatively described as α-D-galactopyranosyl-3-α-D-glucopyranosyl-2-β-D-fructofuranoside.

Table 1. OLIGOSACCHARIDES OF *Lolium* AND *Festuca*

	Sucrose	Raffinose	Other tri-saccharide	Tetra-saccharide
Lolium perenne	+	−	+	−
L. italicum	+	−	+	−
L. temulentum	+	−	+	−
Festuca pratensis	+	−	+	−
F. arundinacea	+	−	+	−
F. rubra	+	+	+	+
F. ovina				
Ssp. *ovina*	+	+	+	+
Ssp. *tenuifolia*	+	+	+	+

+, Present; −, not detected.

The presence of this rather unusual trisaccharide in two members of the Festuceae and its apparent absence from certain other members of the tribe (*Dactylis* and *Cynosurus*)[1] raises the interesting possibility of examining the biochemical affinities of different species of *Festuca* and *Lolium*. It will be recalled that recent morphological and genetical studies of these two genera have cast doubts on their traditional taxonomic alignment. Tutin[4], for example, concludes that *L. perenne* should be transferred to *Festuca*, while Stebbins[5], considering that *F. pratensis* is more closely related to *L. perenne* than it is to other species of *Festuca* such as the type species, *F. ovina*, regards the 'genus' *Lolium* as a section of the large and diverse genus *Festuca*.

The results of chromatographic analyses of the sugars of several species of *Lolium* and *Festuca* are shown in Table 1 and in Fig. 1. All species examined

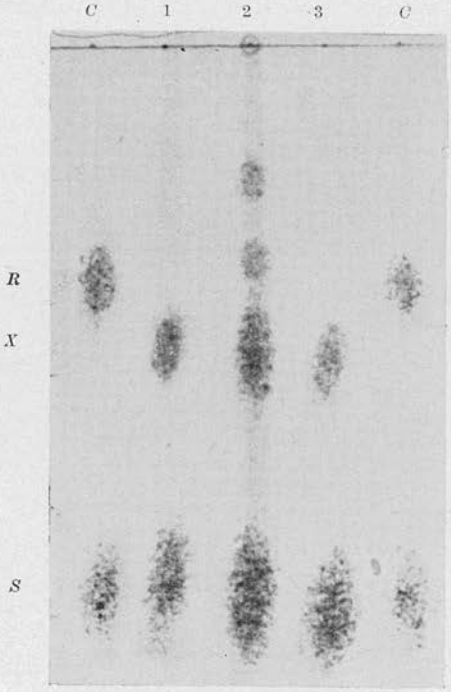

Fig. 1. Oligosaccharides of *Lolium* and *Festuca*. *C*, Control sugars; *R*, raffinose; *S*, sucrose. 1, *Lolium perenne*; 2, *Festuca rubra*; 3, *Festuca pratensis*; *X*, trisaccharide (see text). Solvent: *n*-propanol/water, 78/22

contained the trisaccharide described above, and all three species of *Lolium* gave chromatograms identical with those of *F. pratensis* and *F. arundinacea*. *F. rubra* and *F. ovina*, however, contained two other non-reducing oligosaccharides, both of which yielded galactose, glucose and fructose on hydrolysis. The more mobile of the two was chromatographically identical with raffinose ; the other, which was probably a tetrasaccharide, was not chromatographically referable to stachyose, and calculation of the expression[6] log $[R_F/(1 - R_F)]$ indicated that it might be a higher homologue of the *Lolium* trisaccharide.

The presence of the same rather unusual trisaccharide in all species of *Lolium* and *Festuca* examined is further confirmation from a somewhat unexpected source of the close relationship of the two genera. Moreover, as far as the distribution of sugars is concerned, the affinities of *Lolium* lie with *F. pratensis* and its allies rather than with *F. ovina*, which has a more complicated pattern of oligosaccharide distribution.

We wish to express our thanks to Dr. Mary Noble, Plant Pathology Station, East Craigs, Edinburgh, for a supply of *Lolium temulentum* and to Mr. L. S. Cobley, Edinburgh and East of Scotland College of Agriculture, for samples of *Festuca*.

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CELL WALL METABOLISM.
I. HEMICELLULASES OF *BROMUS* SEEDS

BY ANNA M. MACLEOD AND R. SANDIE

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BLACKWELL SCIENTIFIC PUBLICATIONS
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(With 2 figures in the text)

Summary

Readily extractable hemicelluloses account for approximately 14% of the dry weight of grains of five species of *Bromus*. In *B. mollis*, although 10% of cellulose is present in the whole grain, only one-eighth of this cellulose is located within the true seed, the remainder being restricted to the husk. Crude preparations of enzymes extracted from *B. mollis* can rapidly solubilize and degrade pentosan and hexosan hemicelluloses but are without apparent effect on suspensions of cellulose, and there appears to be no real evidence for the action of a true cellulase during germination.

Extracts of *Bromus* seeds are also extremely active in transglucosylation and can readily catalyse synthesis of β-linked oligosaccharides from 1% solutions of cellobiose.

Introduction

A characteristic and readily observed property of germinating grass seeds is the progressive and often rapid dissolution of the walls of the starch-filled cells of the endosperm. Brown and Morris (1890) attributed this phenomenon to the activities of a cellulase capable of digesting native cellulose, but in a later review (1916), realizing the possible participation of hemicelluloses in wall composition, Brown cautiously avoided any mention of hydrolysis of true cellulose. Other writers have not all been so circumspect, and grass seeds are still not infrequently referred to as sources of 'cellulase'. The proof of this ability to degrade cellulose rests on rather insecure foundations, such as deductions made from the microscopical appearance of sections of endosperm, or measurement of reducing groups liberated from acid-solubilized cellulose by extracts of germinating seeds. However, histological observations do not provide rigid evidence for cellulose degradation if the presence of cellulose has not been fully established, and ability to digest partly degraded material is not necessarily indicative of an equal capacity to hydrolyse native cellulose.

Although cellulose is a constituent of most plant cell walls, there is no *a priori* reason to expect its universal occurrence, and the rather frequent participation of other polysaccharides in endosperm suggests that this tissue may exhibit anomalies in its wall composition as striking as those already established for its cytogenesis. Indeed, from pearled barley, which is 98% endosperm, yields of crude cellulose are sufficient to account only for the walls of the chalazal and transfusion tissue invaginated in the furrow, and there is a high probability that true cellulose is absent from at least the central endosperm (MacLeod and Napier, 1959). Hemicelluloses, however, both hexosan and pentosan, are

certainly present in pearled barley and the pentosan can be shown by suitable histochemical tests to contribute to the cell walls. In barley endosperm the walls are thin and the amounts of hemicellulose present are not great, but certain species of *Bromus* which have thick endosperm cell walls and are liberally provided with water-soluble hemicelluloses (MacLeod and McCorquodale, 1958) afford very suitable material for a critical study of cytolysis in a germinating grass seed.

This problem of cell wall degradation can be attacked both by examining the material believed to contribute to wall structure and by determining the activities of enzymes capable of catalysing their hydrolysis. In the present study methods have been used which were first developed to survey changes in barley during malting (Preece *et al.*, 1952, 1953, 1956, 1958) and before discussing experimental work carried out with *Bromus* it may be useful to summarize the conclusions which have been reached as a result of intensive work with cereal grains.

CEREAL GRAIN POLYSACCHARIDES

In the cereals there is good evidence for the presence of a range of polysaccharides which can be extracted from the ground grain by increasingly drastic procedures. Thus, with barley, after inactivating enzymes and extracting sugars in boiling 80% ethanol, a group of water-soluble components can be brought into solution at 40° C and recovered from the concentrated and filtered aqueous extract by treatment with Fehling's solution and acetone. Not all the water-soluble carbohydrate is removed at this temperature and boiling under pressure causes further solubilization both of starch and of non-starchy polysaccharides. Starch may be eliminated from the extracts by treatment with α-amylase followed by dialysis, and the dextrin-free dialysate affords a second major polysaccharide fraction. Further treatment of the grist with 4% NaOH at room temperature allows extraction of still more hemicellulose which is again purified via a copper complex. These three products — water-soluble, autoclave-soluble and alkali-soluble — are all substantially pure carbohydrate, and they resemble one another in the nature of their principal sugar units — glucose, xylose and arabinose. A pure β-glucan can be separated from the first two by repeated treatment of solutions with 20-30% ammonium sulphate (Preece and Mackenzie, 1952), and the alkali-soluble material yields essentially similar laevorotatory glucan contaminated by some 5% of pentosan (Preece and Hobkirk, 1954).

The highly viscous water-soluble β-glucan has a linear molecule containing approximately equal numbers of 1.3 and 1.4 linkages (Aspinall and Telfer, 1954) and results of controlled acid and enzymic hydrolyses indicate that the different linkages are not arranged in any simple pattern of repeating units (Preece *et al.*, 1960). The presence of β-glucan in each successive extract suggests that the less readily solubilized material is either occluded in the grain by high polymers which themselves are modified by the increasingly drastic extraction procedures or loosely aggregated by means which prevent simple solution but allow extraction after treatment with hot water or with alkali. It seems best to regard these three more or less pure polyglucose carbohydrates as arbitrary sections through a continuum of hemicellulosic glucan rather than as separate entities.

The pentosans of cereal grains present a more complex picture. Araboxylan from barley husk has been examined by Aspinall and Ferrier (1957) and found to resemble straw pentosan: it consists of a backbone of β-1.4-linked xylose residues with arabinose residues principally 1.3-linked, as side chains, and traces of glucuronic anhydride. Structural studies of soluble pentosans from wheat flour indicate that the endospermic

material generally resembles the husk hemicellulose, differing principally in its lack of uronic anhydride and in its higher content of arabinose residues; it has been suggested (Perlin, 1951) that transarabinosylation mechanisms may be important in introducing extra arabinosyl groups, increasing solubility, and so making possible some translocation of the pentosan molecules.

Although it is simple to prepare pure β-glucan from various cereal sources it is less easy to separate pentosan from the α-linked dextrin which has precipitation characteristics similar to those of the pentosan, and up to the present the only satisfactory source of a reasonably pure cereal pentosan has been rye extracted with water at 20° C. These two products — β-glucan from barley and pentosan from rye — have proved very useful as substrates for the study of hemicellulases.

Hemicellulases of cereal grains

As far as β-glucan degradation is concerned, there are present in barley (*a*) endoglucanase which catalyses internal scission of the long β-glucan molecule; (*b*) exoglucanase which removes oligosaccharides from chain ends; and (*c*) cellobiase (Preece and Hoggan, 1956). Endoglucanase activity is readily measured by recording the fall in viscosity of solutions of β-glucan in presence of the enzyme; exoglucanase activity is shown by estimating reducing groups produced in excess of those attributable to endo-enzyme; and cellobiase is determined with dilute solutions of cellobiose as substrate. In this last determination it is important to examine the reaction mixtures for transglycosylation, which may on occasion be extensive enough to obscure hydrolysis. Using these methods it has been shown that the common cereals contain different proportions of the different components of the β-glucan-degrading system, and that substantial changes in the relative amounts of the major enzyme components occur as seedling growth proceeds.

As was the case with the characterization of pentosans, so has the determination of pentosanases proved rather troublesome. It is now clear, however, that among the enzymes concerned are an endoxylanase, an arabinosidase capable of stripping off individual arabinose units, an exoxylanase which can apparently come into action only after impeding arabinosyl groups have been removed, and a xylobiase (Preece and MacDougall, 1958). Though there are obvious analogies between the pentosanases and the glucanases, comparison of activities at various stages of seedling growth indicates that two separate groups of enzymes are involved, and not merely two facets of the generalized action of a series of glycosidases of low specificity.

With this rather formidable background of information available for cereal grains, the present studies on cell wall degradation of *Bromus* endosperm were initiated in the hope of elucidating the pattern of cytolysis in a native grass seed, and, if possible, relating observations made *in vitro* to events known to take place in the intact germinating seed.

Experimental

Cell wall polysaccharides of Bromus

Five species of *Bromus* were used: *B. erectus* Huds., *B. inermis* Leyss., *B. mollis* L., *B. ramosus* Huds. and *B. sterilis* L. Hemicelluloses were successively extracted from enzyme-inactivated grain (*a*) in water at 40° C; (*b*) in water at 100° C after autoclaving at 120° C and (*c*) in 4% NaOH at room temperature. With the exception of *B. erectus*, of which only 10 g of grain was available, all determinations were made in duplicate on at

least 20 g of grain. Results of these polysaccharide estimations are given in Table 1, and the proportions of the constituent sugar units, determined after hydrolysis, chromatographic separation of the hydrolysates and estimation of the individual sugars by Somogyi (1945) titrations, are shown in Table 2.

Table 1. *Hemicelluloses of* Bromus *seeds*

Species	(grammes per 100 g dry weight) Soluble at 40° C	Autoclave-soluble	NaOH-soluble	Total
B. erectus	2.30	4.29	9.18	15.77
B. inermis	2.42	3.15	7.85	13.42
B. mollis	1.23	7.10	6.91	15.24
B. ramosus	2.30	4.15	7.73	14.18
B. sterilis	1.97	6.10	5.57	13.64

A highly viscous β-glucan of specific rotation $\alpha[D] = -10°$ could readily be prepared from the water-soluble and the autoclave-soluble polysaccharides by addition of ammonium sulphate to 30% saturation, and this β-glucan accounted for approximately 70% of the total glucose in the water-soluble material; no real success was achieved in separating pure polymers from the alkali-soluble fraction, though differential precipitation with acid and acetone secured substantial enrichment of the products with respect to either glucan or pentosan. *Bromus* β-glucan thus generally resembles barley β-glucan in its solubility characteristics and specific rotation and in the extent to which it can be freed from contaminants, though the quantities found in the grain are very much greater than those in the barley corn.

Table 2. *Composition of* Bromus *polysaccharides*

	Fraction and anhydrosugar composition*								
	Water-soluble (40°C)			Autoclave-soluble			NaOH-soluble		
	G	X	A	G	X	A	G	X	A
B. mollis	55	25	20	72	19	9	40	47	13
B. ramosus	28	50	22	35	36	29	41	42	17
B. sterilis	53	29	18	48	36	16	44	37	19

*G = glucose, X = xylose, A = arabinose, all as % of product.

The figures quoted in Tables 1 and 2 are interesting in that they demonstrate the presence of some 15% of total extractable hemicelluloses in various species of *Bromus*, but it cannot be inferred from these figures that this polysaccharide mixture is inevitably of importance in germination, for its distribution within the grain is uncertain. Thus the husk investing the caryopsis is not implicated in carbohydrate transformations, and analysis of husk-free material was therefore desirable. Mechanical removal of husk was impracticable, but immersion in 50% H_2SO_4, as recommended for eliminating barley dormancy (Pollock *et al.*, 1955) achieved a clear separation of husk from the grain of *B. mollis*. With *B. sterilis* the technique was less successful as the loosened fragments of husk remained inextricably mixed with the rest of the grain and could not be washed off. Germinative capacity was unimpaired by the acid treatment and, indeed, the virtual elimination of micro-organisms from the seed surface was highly advantageous.

The sample of *B. mollis* used was collected from the same locality as that examined for the data of Tables 1 and 2 but was of a different season's growth. Analyses of the whole grain and of the husk-free material prepared from a 25-g sample of grain were carried out

by the methods described above, and results (Table 3) are given for 100 g of original grain, including husk, in each case. Removal of husk (20% of the dry weight of the sample) was accompanied by no significant decrease in water-soluble polysaccharides, but there was a distinct loss, presumably to the husk which was discarded, of alkali-soluble material, particularly xylan. It may be noted in passing that the initial treatment with acid was not wholly without effect on the grain carbohydrates for, rather surprisingly, the arabinose contents of the autoclave-soluble and the alkali-soluble hemicelluloses were regularly higher than those from the untreated grain. This anomaly remains unexplained.

Table 3. *Polysaccharides of* B. mollis

Polysaccharide	Yield in grammes per 100 g of original grain (seed + husk)			
	Whole grain	Husk-free seed	Loss (to husk)	
Water-soluble (40° C)	1.30	1.27	0.03	
Autoclave-soluble	7.26	6.02	1.24	(mainly glucan)
NaOH-soluble	7.75	5.24	2.51	(equal amounts of glucan and xylan)
Crude cellulose	10.25	1.25	9.00	

Crude cellulose was also determined in both intact and husk-free *B. mollis* using the method of Norman and Jenkins (1933): results of these analyses are also given in Table 3. The low concentration of cellulose in the husk-free grain is noteworthy and is reminiscent of the condition already established for barley (MacLeod and Napier, 1959). Despite the drastic treatments involved in cellulose purification, the final product closely resembles the cell walls from which it was derived, and comparison with macerated fresh tissue can frequently suggest the origin of the final cellulose. Microscopic examination of cellulose from husk-free *B. mollis* showed spindle-shaped interlocking fibres resembling the walls of the outermost layers of the caryopsis; little amorphous material was present and there were no structures at all similar to the walls of the starch-filled endosperm cells.

The results shown in Tables 1-3 indicate that all five species of *Bromus* contain very substantial amounts of polysaccharide of a hemicellulosic nature, with, generally, approximately equal amounts of hexosan and pentosan, and of varying degrees of solubility. In *B. mollis*, which has been most fully investigated, there is eight times as much hemicellulose in the husk-free grain as there is cellulose; it may be noted, also, that the figures for hemicelluloses are minimal since losses are inevitable in preparing pure polysaccharides by methods involving repeated solution and precipitation, whereas the figure for cellulose is maximal as it represents a final residue with no correction for ash or other impurities.

The procedures outlined above do not account for all possible hemicellulosic material in a seed, for polysaccharides are known which are not easily solubilized by alkali but which are lost during the series of treatments with hypochlorite and sulphite entailed in purifying cellulose. No satisfactory method has yet been found for extracting this category of hemicelluloses in a state resembling that in which they occur in the cell walls, though it can be inferred from results of determinations made with germinating seeds that xylose contributes to their structure.

Hemicelluloses and seedling growth

Although it is possible to provide results for the balance of various hemicellulose fractions during seedling growth, certain obvious complications arise. Thus, with the increasing production of dextrins from starch, direct determination of water-soluble polysaccharides

is of little value, and it is necessary to estimate authentic β-glucan precipitable with 20% ammonium sulphate and showing a specific rotation of approximately −10°. Moreover, the active synthesis of new cell wall material soon introduces major complications into any investigation of purely degradative changes, and results afford little more than an indication of possible transformations unless separation of seedling from endosperm is possible. The types of change indicated here are thus principally of value, not in providing data on which to build elaborate hypotheses, but rather in suggesting types of enzyme system which should be investigated.

For germination 25 g of *B. mollis* were freed from husk and the caryopses were maintained on moist filter paper at 25° C in the dark: this temperature was found to result in maximum germination and regular growth. About half of the seeds showed signs of growth beginning in 2-3 days and in a further 30% rootlets had emerged by the fourth day; the remaining 20% failed to grow but showed no signs of mould. This freedom from micro-organisms was in strong contrast with the state of affairs in grain which had not been treated with acid, where both *Alternaria* sp. and a motile rod-shaped bacterium were much in evidence. It was not practicable to remove the ungerminated seeds from the growing plants and results are given for the total bulk.

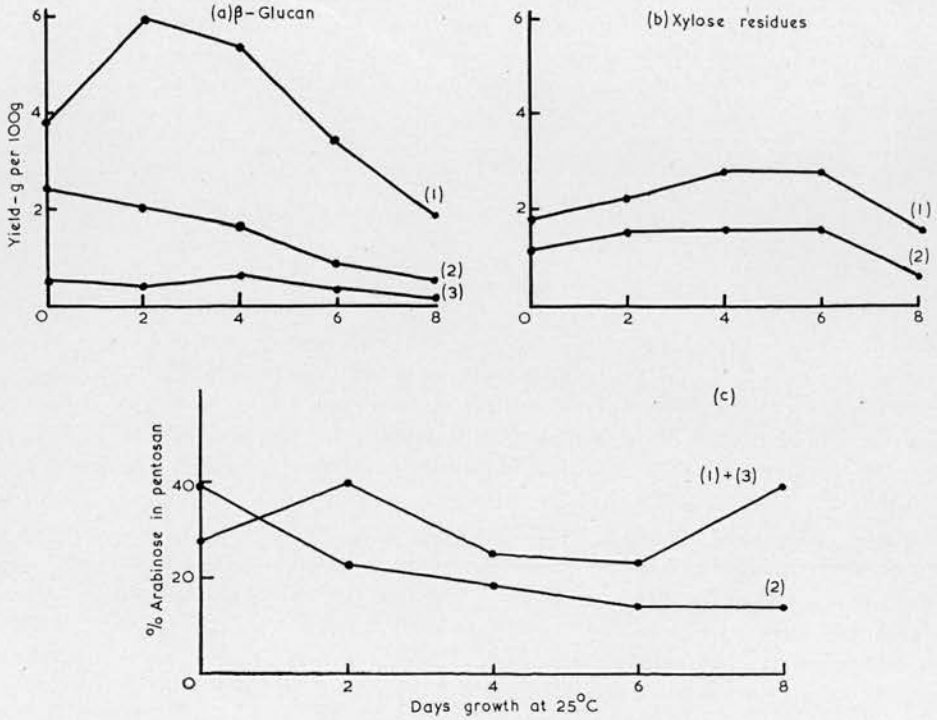

Fig. 1. Changes in β-glucan and pentosan of *B. mollis* during seedling growth. 1 = autoclave-soluble polysaccharide; 2 = alkali-soluble polysaccharide; 3 = water-soluble polysaccharide.

It will be seen from Fig. 1a that while alkali-soluble glucan diminished throughout the experimental period there was first a marked increase in the autoclave-soluble material, so that total extractable glucan was present in larger amounts in the fully imbibed seed. It is highly improbable that extensive synthesis of cell wall polysaccharides is taking

place at this time and it seems likely that purely physical changes within the grain are conducive to greater ease of extraction. Ignoring for the present this initial rise and concentrating on the overall changes, it appears that total β-glucan falls from a 2-day maximum of over 8% of the seed to a level of 2.3% at 8 days: a glucanase system capable of degrading the polysaccharide to a degree of polymerization below that at which precipitation takes place would seem to be implicated in this change. Fig. 1b shows changes detected in total xylose residues of autoclave-soluble and alkali-soluble pentosan; no results are given for water-soluble xylan since changes in this fraction were insignificant. Again, a pattern of increasing extractability is apparent with eventual diminution in amounts of recoverable material, leading to a final recovery of three-fifths of the xylose residues accounted for at the point of maximum extraction. In addition to this apparent degradation of xylan, there is a simultaneous alteration in the proportion of arabinose residues associated with the xylan (Fig. 1c). The alkali-soluble pentosan is at first depleted of arabinosyl units while the two more soluble fractions are enriched in arabinose during the first 2 days, and then behave in a manner similar to that noted for the alkali-solubles. The final proportionate increase in arabinose in the water-soluble fraction accompanies a period of rapid seedling growth, and may reflect the synthesis of a range of hemicelluloses in the coleoptile and rootlets, with pure araban and assorted araboxylans all playing their parts. The vicissitudes undergone by the pentosan are too complex to allow of any useful discussion at present, but again it is clear that both xylan-degrading enzymes and arabinosidases are likely to be active at an early stage in seedling development.

Hemicellulases

Preparation of enzymes. The methods used were essentially those of Preece and Hoggan (1956). 30 g of finely ground grain of *B. mollis* was extracted with 200 ml of 0.6% NaCl for 1 hour at room temperature and the clear centrifuged extract was allowed to stand overnight and then dialysed against running tap water for 2 days. Four volumes of acetone at 4° C were added and the separated precipitate was redissolved in water, reprecipitated and dried with increasing strengths of cold acetone. The initial overnight stand allowed digestion of accompanying polysaccharide by the extracted enzymes and thus no interfering carbohydrate was introduced into later enzyme assays. Although some autolysis of enzyme must also have occurred this disadvantage was heavily outweighed by the advantages of using a polysaccharide-free enzyme preparation. Yields of crude enzyme were of the order of 1% of the grain.

Substrates. (1) Pure β-glucan was prepared from barley (Preece and Mackenzie, 1952) and satisfactory samples had the following characteristics: $\alpha_{[D]}$ −12°; reducing power not more than 2.5 μg per mg; specific viscosity $\eta_{sp}^{25°}$ not less than 5 for a 0.5% solution; and hydrolysis product, only glucose. Ideally it would be desirable to use β-glucan from *Bromus* as substrate, but the collection, cleaning and drying of kilogramme quantities of grass seed was impracticable, and the polysaccharide from barley had sufficiently similar properties to serve as an adequate substitute.

(2) Pentosan was prepared from rye by fractionating the water-soluble polysaccharide mixture with ammonium sulphate and retaining only the material which precipitated between 40 and 50% saturation. This fraction had $[\alpha]_D$ −135°; reducing power of 2.5 μg per milligramme, specific viscosity (0.5% solution) $\eta_{sp}^{25°} = 6.0$. The ratio of xylose to arabinose was 60 : 40 and some 3-4% of glucan was also present as an undesirable but apparently irremovable contaminant.

(3) The alkali-soluble hemicellulose mixture from husk-free *B. mollis* (Table 3) was used without further purification.

(4) The cellulose from husk-free *B. mollis* was macerated and used as an aqueous suspension. This sample of crude cellulose appeared to be a pure glucose polymer as only glucose was detected chromatographically after solution in 72% acid and subsequent hydrolysis with dilute acid.

Measurement of enzyme activity

(1) *Transformations of soluble β-glucan.* Endo-β-glucanase activity was determined viscometrically in precisely the same manner as was used for cereal enzymes (Preece and Hoggan, 1956) and each result is quoted as increase in reciprocal specific viscosity per milligramme enzyme per hour, with a reaction mixture of 8 ml of 0.5% glucan, 1 ml of buffer pH 5.0 and 2 ml of enzyme solution. Results of typical determinations are shown in Fig. 2a.

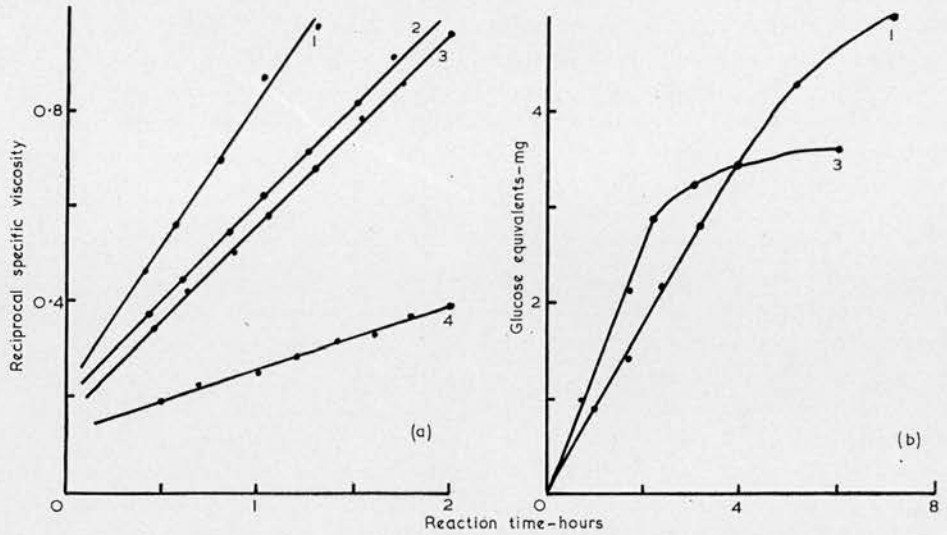

Fig. 2. Typical results of determinations of (a) endoglycosidase (b) reducing group production. 1, 2 and 3 were glucan substrates with enzyme preparations from different samples of *B. mollis*; 4 was a pentosan substrate.

For determination of exo-β-glucanase activity the reaction mixtures were of the same proportionate composition as those used in the viscometric method. Incubation was at 25° C and 5-ml portions were withdrawn at intervals and boiled to terminate enzyme action before estimating reducing activity by Somogyi (1945) titrations. The increase in reducing power is attributable to the joint action of endo- and exo-enzyme, but correction for reducing groups produced by endoglucanase can be determined from the equation $g = 1.165n \pm 0.012$, where g: represents milligrammes glucose and n = increase in reciprocal specific viscosity due to endo-enzyme. Results are calculated from the linear portion of the graph in Fig. 2b and are presented in terms comparable with those for endoglucanase.

The calculated final figure is, however, only a best estimate of degradative exoglucanase, for the oligosaccharides produced are in a state of flux, subjected on the one hand to cellobiase action and on the other to transglycosylation with synthesis of larger β-linked

oligosaccharides. The departure from linearity after 2-4 hours (Fig. 2b) suggests increasing transferase action as increasing numbers of free end groups are made available by the action of the enzyme mixture.

As regards cellobiase activity, a preliminary survey using conditions previously established as suitable for cereal enzymes suggested that cellobiase was absent from *Bromus* preparations, since no increase in reducing power was apparent when enzyme and cellobiose were incubated for periods ranging from 1 to 7 hours. However, chromatographic examination showed that extensive action, both hydrolytic and transglycolytic, had in fact occurred during the first hour, and only after dilution of the substrate to 0.05% was it possible to estimate hydrolysis without obvious interference from concomitant synthesis.

Results of typical determinations of various aspects of glucanase action are summarized in Table 4 along with comparative results for barley and for oats.

Table 4. *β-glucanase activity*

	Endoglucanase		Exoglucanase		Cellobiase	
	Activity*	Activity × yield†	Activity‡	Activity × yield	Activity§	Activity × yield
B. mollis (1957)	0.180	144	0.267	213	0.82	656
B. mollis (1958)	0.130	130	0.162	160	0.76	760
Oats	0.0225	14	0.002	2	—	208
Barley	0.0114	8	0.022	15	0.8	572

*Increase in reciprocal specific viscosity per milligramme enzyme per hour.
†Yield of enzyme in milligrammes per 100 g grain.
‡Reducing groups produced, as glucose, corrected for action of endoglucanase, per milligramme enzyme per hour.
§Milligrammes glucose produced from cellobiose per milligramme enzyme per hour.

(2) *Transformations of soluble pentosan.* Using methods and reaction mixtures similar to those described for determinations of endoglucanase activity, but with soluble pentosan from rye as substrate, measurements were made of viscosity diminution attributable to endopentosanase (Fig. 2a) and chromatographic separation served to ascertain the rate of oligosaccharide and sugar production. The branched nature of the substrate molecule makes pentosan breakdown less amenable to mathematical treatment, and, although reducing group production has been followed over a period of hours, no correction can as yet be applied for endopentosanase, and only general results are quoted (Tables 5 and 6); these again are supplemented by figures derived from similar work with barley.

Table 5. *Pentosanase activity with soluble substrate*

	Endoxylanase activity	Activity × yield*
B. mollis	0.032	32
Oats	0.027	10
Barley	0.013	4

*See footnote to Table 4.

(3) *Transformations of alkali-soluble hemicelluloses.* Suspensions of the alkali-soluble mixed polysaccharide from husk-free *B. mollis* were incubated with enzyme in the usual proportions at 37 °C. Reducing groups were determined at intervals on aliquot samples chosen so that any changes observed were due to precisely 1 mg of enzyme, and total hexose and pentose were determined after acid hydrolysis of similar aliquots. All acid

hydrolysates gave identical results (as would be expected) so the homogeneity of the suspension was judged to be satisfactory; the extent of enzymic hydrolysis achieved is shown in Table 7. Chromatographic examination of the incubation mixtures revealed five separate groups of higher oligosaccharides in addition to mono- and di-saccharides. One oligosaccharide corresponded to xylotriose, others appeared to contain varying numbers of glucose residues and all except the least mobile were apparent after 6 hours' incubation. No precisely similar work has been reported with cereal enzymes, though a preliminary survey with barley suggests a very meagre capacity to attack alkali-soluble hemicelluloses.

Table 6. *Sugar and oligosaccharide production from soluble pentosan. Chromatographic separation was in Butanol: Acetic acid: Water* (40 : 10 : 50, *top layer*)

Chromatographic mobility (R_F)	Period of enzymolysis in hours*					Tentative identity
	B. mollis		Barley			
	1	8	1	16	40	
0.003	—	+	—	—	+	Hexasaccharide
0.008	+	+	—	—	+	Pentasaccharide
0.02	+	+	—	—	+	Tetrasaccharide
0.05	—	+	—	—	+	Xylotriose
0.11	—	+	—	+	+	Xylobiose
0.23	+	+	+	+	+	Arabinose
0.26	—	+	—	+	+	Xylose

*+ = PRESENT; — = NOT DETECTED.

(4) *Transformations of cellulose.* After 6, 24 and 48 hours' incubation of finely divided cellulose with enzyme at 37° C, aliquots of the suspension were removed and examined both titrimetrically and chromatographically. An increase in reducing power, calculated as glucose, equivalent to 4% total hydrolysis of the substrate was recorded after 6 hours and no further increase was detected on more prolonged incubation. Chromatographic examination of the filtered concentrated mixture showed the presence of xylose, arabinose, glucose, galactose and xylobiose. These sugars must have been derived from the 'cellulose' since the enzyme control was blank, and it must be concluded that the cellulose was not, as first thought, a pure glucose polymer. The five sugars gave reactions of equal intensity when the chromatograms were sprayed, so it seems that the maximum production of glucose from this substrate cannot have amounted to more than 1% in 48 hours. Finally, an acid hydrolysate of a filtered 48-hour digest showed slight intensification of the xylose on the chromatogram, but no apparent increase in glucose. It can safely be concluded that hydrolysis of true cellulose was negligible.

Table 7. *Action of enzyme from* B. mollis *on alkali-soluble hemicellulose*

Length of treatment (hours)	Reducing groups (mg. glucose)	Enzymic hydrolysis (% of total)
6	1.2	5
24	4.4	19
48	9.8	43
Acid hydrolysis	22.8	—

The results described above were all obtained with enzyme preparations from ungerminated seeds, but no essential change in the type of enzyme present was observed when growth continued till the endosperm was exhausted, though there was a general enhancement of all aspects of glucan degradation.

Discussion

It can be seen from the results presented above that *Bromus* seeds are liberally provided with β-linked pentosans and hexosans and with a wide range of assorted β-glycosidases capable of catalysing the partial or complete degradation of both soluble and insoluble polymers. The amount of cellulose present in the true seed is insignificant and even with the most active enzyme preparations available true cellulose was not appreciably attacked. If, as now postulated, the carbohydrates of the endosperm cell walls are exclusively hemicellulosic, and extracts of the grain yield enzyme systems sufficiently powerful to degrade these hemicelluloses, then it should be possible to observe cell wall dissolution when sections of endosperm are incubated with appropriate enzyme preparations. When this question was first examined results were disappointing, and little visible change could be detected in sections incubated for periods of up to 48 hours in presence of either enzymes extracted from ungerminated seed, as described above, or similar enzyme mixtures of even greater activity prepared from material which had grown for 2-4 days. However, inadvertant excess pressure applied to a cover glass eventually revealed that the cell walls, though still present, were structually greatly altered, for very slight pressure caused them to disperse, so that starch grains were liberated into the surrounding medium, leaving a reticulate matrix of granular material which originally occupied the spaces between the starch grains, together with flocculent deposits derived from the walls. In sections which had similarly been maintained in sterile water the cell walls remained firm, showing no signs of collapse even after rather heavy pressure or considerable torsion. The composition of the residual material — which, incidentally, is not present in exhausted endosperms attached to growing seedlings — is now being studied.

Although this investigation was principally designed to survey the means by which *Bromus* seeds achieve solubilization of their endosperm cell walls, a number of side issues have arisen which are worthy of some comment. Most interesting of these is, perhaps, the powerful transferase activity shown by crude enzyme preparations acting on dilute solutions of cellobiose. Ever since the first reports of synthesis by invertase preparations (Edelman and Bacon, 1951) records have accumulated of transglycosylations performed by enzymes which are essentially hydrolytic in their action on dilute substrates. Active transferase is usually detected with concentrated substrate solutions, Anderson and Manners (1959), for example, using 20% cellobiose, were able to demonstrate synthesis of two β-linked oligosaccharides in 48 hours in presence of barley β-glucosidase. With *Bromus* enzymes, however, incubation with 1% cellobiose for 5 hours yielded six distinct separate carbohydrate components in addition to cellobiose and glucose, and with R_F values (butanol: acetic acid: water, 50 : 10 : 40) ranging from 0.002 to 0.24. Phosphate appeared to be implicated in the structure of some, though not all, of the oligosaccharides, and chromatographic mobilities of some, though again not all, of the oligosaccharides corresponded, in various solvents, to those observed during the very early stages of β-glucan degradation. Attempts to characterize the individual oligosaccharides are now in progress, and the notable activity of seeds of this genus of grasses in transglycosylation suggests exciting possibilities of exploring the mechanism of synthesis of fibrous β-linked polymers of glucose in an Angiosperm.

Other facets of polysaccharide degradation by *Bromus* generally resembled events already established for barley (see Preece, 1957) though it has as yet proved impossible to obtain, by inhibitor treatment, the preparations of endoglucanase free from exo-activity

which have been so valuable in exploring partial breakdown of β-glucan and so determining its structure.

It is reasonable to suppose that malting barley has been unwittingly selected for thinness of endosperm cell walls, together with maximum content of starch, since the making of malt is largely a matter of 'modifying' these walls to yield a friable malt corn, and it is interesting, though not really surprising, to find that similar types of mechanism are employed in mobilizing the reserves of an exceptionally thick walled grass seed and in securing the relatively small degree of polysaccharide solubilization desired in malting. In neither case, however, is it necessary to invoke the presence of a cellulase to explain the physiological and structural changes involved in germination.

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EFFECTS OF GIBBERELLIC ACID ON BARLEY ENDOSPERM

BY

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When slices of barley endosperm are incubated with gibberellic acid in aseptic conditions the activities of salt-soluble endo-β-glucanase and endopentosanase are greatly augmented and enzymes and soluble products of cell-wall degradation (hexosan and pentosan) are secreted into the surrounding medium. This cytolytic action, together with the enhanced amylolytic activity which can be detected in isolated endosperms subjected to gibberellic acid, is sufficient to cause complete solubilization of the starchy endosperm in 3 days at 25° C. Gibberellic acid acts through the respiring aleurone layer; it has no effect on aleurone-free endosperm or on endosperm slices in which the aleurone has lost the power of reducing tetrazolium salts. Gibberellic acid-induced sugar secretion from endosperm slices is maximal at 30° C. and completely inhibited at 37° C. and secretion both of sugars and of endo-β-glucanase is unaffected in conditions which inhibit proteolysis. Excised barley embryos show no response to added gibberellic acid. It is suggested that, when barley germinates without additions of gibberellic acid, endogenous gibberellin present in the embryo is translocated to the aleurone where it induces secretion of hydrolytic enzymes from subcellular particles, so causing modification to proceed inwards from the aleurone; if this is so, then added gibberellic acid merely enhances the effects of the endogenous component.

Introduction

There is now no doubt that controlled treatment of malting barley with gibberellic acid can result in increased levels of activity of many of the hydrolytic enzymes of the finished malt. Enhanced activities of proteinase, α-amylase, and β-hydroxymethylcellulase in treated malts are well authenticated[9,20,21] and, concomitant with the rise in enzymic potential, there is an increase in the speed of modification of endosperm cell walls and a concurrent economy in malting time. Malts from gibberellic acid-treated barleys frequently give higher extract yields than those from control samples which have been grown until modification is judged to be complete,[12] and this extra extract is at least partially attributable to an increased concentration of fermentable sugars in the wort. These effects are naturally of very great practical importance for the malting industry, and it is obviously desirable to attempt to understand the fundamental biochemical mechanisms responsible for the gross changes detectable in the analytical characters of malts made from gibberellic acid-treated grain.

Attempts to trace to their origins changes induced by alteration of a single environmental factor which is known to influence the development of a grain of barley are fraught with difficulty: the metabolic reactions involved in germination are all interrelated, and it is not easy to distinguish direct primary effects caused by altering one experimental variable from the many interacting secondary effects which are consequent upon the primary one. Thus, to take a simple example, when gibberellic acid is added to malting barley two of the changes regularly observed, *viz.* increased proteolysis and increased production of α-amylase, represent effects on two distinct enzyme systems, and it is relevant to enquire, *e.g.*, whether the enhanced amylolytic powers are caused by increased proteolysis giving greater extractability of other enzymes, whether both groups of enzymes are stimulated directly by gibberellic acid, or whether both are influenced by some third mechanism which in turn responds directly to the presence of gibberellic acid.

Again, in attempting to uncover the fundamental effects caused by adding an

unfamiliar component to a biological system, the choice of experimental material would normally be a reasonably homogeneous tissue such as a callus culture or a root meristem, and any results could then at least be related to the behaviour of one type of cell. A barley grain is a most unsuitable experimental object: the embryo contains two regions—the acrospire and the rootlets—which are both capable of growth but which respond quite differently to gravity and to light, and a third—the scutellum—which does not grow but whose outer epithelial layer exhibits considerable powers of enzyme secretion.[3] The endosperm is also a complex organ: the two or three outermost layers of cells, the aleurone, are rich in lipids and in proteins and are capable of reducing tetrazolium salts, whilst the central endosperm is a non-respiring mass of starch-containing cells with atrophied nuclei and, apart from their content of β-amylase,[6] with little known capacity for metabolic activity. This heterogeneous association of cell types is enclosed in a selectively semipermeable membranous pericarp-testa and is surrounded by an inert husk, and it is on one or more of these tissues that gibberellic acid exerts its influence.

Gross general analyses of treated malt, valuable though they are to the maltster and to the brewer, do not shed much light on possible mechanisms of action of gibberellic acid since they cannot give any indication even of the probable site of action of the additive. However, studies recently carried out in Japan by Yomo[24] and in Australia by Paleg[14,15] have shown quite unequivocally that gibberellic acid can act directly on barley endosperm in the absence of the embryo. In the Japanese work, embryos were excised from dehusked grain and the residual endosperms were sterilized in bromine water and then incubated in various concentrations of gibberellic acid: control material maintained in water produced 17 units of α-amylase whereas treated endosperms gave 600–900 units of this enzyme. Paleg has confirmed and amplified these observations, and has been able to show that the enhanced amylolytic activity is reflected in an excretion of reducing sugars to the medium surrounding the endosperms.[14] Paleg has further demonstrated that gibberellic acid treatment of isolated endosperms not only augments β-amylase content and initiates formation of α-amylase, but it also induces formation of an amylolytic agent where characteristics closely resemble those of R enzyme.[15] This last observation is of considerable importance, as it links up with the findings of Kringstad *et al.*[10] and of Ault,[1] who have detected proportionately larger amounts of maltotriose + simpler sugars, in comparison with dextrins, in worts produced from gibberellic-acid treated barleys.

Both Yomo and Paleg have been concerned mainly with amylase production; their general technique, however, is well suited to an investigation of cytolytic enzymes, and the work reported here has been concerned both with the general phenomenon of induction of enzyme activity in isolated endosperm fragments and with the specific question of the influence of gibberellic acid on the β-glucanases and pentosanases which are involved in carbohydrate modification in the endosperm.

Experimental

Site of Action of Gibberellic Acid

One of the principal hazards of working with barley grains is a microbiological one. Lactobacilli and other organisms are regularly present between the husk and the pericarp-testa, and sterilization of intact grain is not easy to secure without impairing the germinative capacity of the seed. Although Paleg found that a preliminary treatment with hypochlorite, followed by exposure to dilute streptomycin in the incubation medium, gave sterility over the 24 hr. which his experiments occupied, we were reluctant to use streptomycin as there seems to be no real understanding of its mode of antibiotic action; there is moreover a possibility that interaction between gibberellic acid and streptomycin might introduce irrelevant and possibly undetected complications. It was found, however, that barley which had been dehusked by treatment with 50% sulphuric acid carried no viable micro-organisms, and careful attention to aseptic technique in subsequent manipulations allowed endosperm slices to be maintained for periods of up to 72 hr. without any development of micro-organisms. As a check on sterility, aliquots of the experimental material were incubated on glucose-agar at the conclusion of the trials, and any contamination was noted.

General technique.—Slices of endosperm, approx. 2 mm. thick, were cut transversely

from barley which had been dehusked by means of sulphuric acid and subsequently washed in sterile water. 10–15 such slices were placed in a sterile petri dish either in 4 ml. of water or in a solution of gibberellic acid; after incubation, the ambient fluid and —in some instances—the residual endosperm slices were analysed for reducing sugars or for β-glucanases or pentosanases. Determinations of reducing sugars were made by the Somogyi[23] method, and β-glucanases and pentosanases were estimated by the methods of Preece *et al.*[17,18] No corrections have been made for the moisture content of endosperm slices, which was of the order of 12%.

Liberation of sugars from endosperm slices.—Preliminary work with slices cut from Wisa barley showed that gibberellic acid at concentrations between 10^{-2} and 50 μg. per ml. of surrounding fluid induced secretion of reducing sugars into the medium (Table I).

TABLE I

EXCRETION OF REDUCING SUGARS FROM ENDOSPERM SLICES

(22 hr.; 21°C.)

Gibberellic acid (μg. per ml. of ambient fluid)	Reducing sugar (as glucose) (mg. per 100 mg. of endosperm)
0	0·3 (±0·027)*
0·001	0·3
0·01	0·4
0·1	1·4
0·3	1·9
0·6	1·8
1·0	1·7 (±0·16)*
2·0	1·5
3·0	1·6
5·0	1·7
10·0	1·6
25·0	1·7
50·0	1·6 (±0·15)*

* Means and standard deviations of 10 determinations. The other results are means of two determinations.

No real optimum value for gibberellic acid concentration could be observed, and the figures for the release of reducing sugars are of the same order as those quoted by Paleg[14] for Prior barley subjected to gibberellic acid concentrations between 2 and 200 μg. per 3 ml. of surrounding fluid. Analyses of 80% ethanolic extracts prepared from the residual slices by boiling under reflux showed that, in the gibberellic acid-treated material, reducing sugars amounting to between one-quarter and one-fifth of the total excreted into the medium were retained in the tissues: the untreated slices contained similar proportions of reducing sugars.

Influence of aleurone.—Cubes of starchy cells free from aleurone were cut from the centre of endosperm slices. 100-mg. portions of the aleurone-free material and similar amounts of the residual peripheral fragments containing aleurone were incubated separately either in water or in presence of gibberellic acid, at concentrations ranging from 10^{-2} μg. per ml. to 100 μg. per ml. The amounts of sugars released are shown in Table II, where

TABLE II

SUGAR RELEASE FROM ALEURONE-FREE ENDOSPERM AND FROM ENDOSPERM WITH ALEURONE

Gibberellic acid (μg. per ml. of ambient fluid)	Reducing sugar, as glucose* (mg. per 100 mg. of tissue)	
	Aleurone-free endosperm	Endosperm with aleurone
0	0·12	0·4
0·01	0·14	0·5
0·1	0·12	4·0
1·0	0·14	3·6
10·0	0·13	3·2
100·0	0·11	3·8

* All results are means of duplicate determinations.

it can clearly be seen that the presence of aleurone is essential to the success of the gibberellin-induced sugar release. Again, no optimum value for gibberellic acid concentration could be observed, and in subsequent work gibberellic acid was supplied at a level of 5 μg. per ml. This is a high level of application compared with the concentrations used in malting practice, but it was well within the range of efficacy of the additive and it proved convenient to use without the necessity for extensive dilution.

Progressive sugar release with time.—Eight replicates of sets of endosperm slices were prepared; 4 were incubated at 25° C. in presence of gibberellic acid, four were maintained in water as controls, and one of each series was tested at appropriate intervals for presence of reducing sugars in the ambient liquid. Results of a typical set of determinations are given in Table III. In the example quoted, the contents of all eight petri dishes remained sterile for the duration

of the experiment; in other trials where bacterial contamination was noted the same general pattern of sugar release was apparent,

TABLE III

PROGRESSIVE RELEASE OF REDUCING SUGAR FROM ENDOSPERM SLICES

Length of incubation (hr. at 25° C.)	Sugar release (mg. apparent glucose per 100 mg. of tissue)	
	Control (water)	Gibberellic acid
17	0·5	1·1
20	0·6	2·6
48	0·9	36·0
72	1·0	67·0

though the apparent concentration of reducing sugar was lower—*e.g.* 20 mg. per 100 mg. instead of 36 mg. detected in uninfected experiments after 48 hr. This reduction in apparent sugar output was doubtless the result of consumption by the invading micro-organisms. Dissolution of the starchy endosperm began at the sub-aleurone layer and progressed inwards; after 48 hr. the aleurone had begun to separate from the underlying layers in the sections which were exposed to gibberellic acid; after 72 hr. these slices were virtually emptied of solid contents, apart from the aleurone itself which retained its walls and much of its apparent structure. Chromatographic examination of the exudates from the slices revealed the presence of a wide range of sugars and oligosaccharides, and, as far as was possible, the various

TABLE IV

CHROMATOGRAPHIC IDENTIFICATION OF SUGARS AND OLIGOSACCHARIDES EXCRETED FROM ENDOSPERM

(72 hr. incubation at 25° C.)

	From hexosan		From pentosan
	α-linked	β-linked	
Monosaccharide	Glucose		Arabinose Xylose
Disaccharide	Maltose	Cellobiose Laminaribiose	Xylobiose
Trisaccharides	Maltotriose	Cellotriose (?)	Xylotriose (?)
Other oligosaccharides	4 Uncharacterized		3 Uncharacterized

chromatographically-distinct components were compared with known materials and tentatively identified (Table IV). In contrast to the gibberellic-treated slices, the control material liberated to the medium only glucose, maltose and a trace of maltotriose.

It is abundantly clear that gibberellic acid has stimulated not only amylolytic activity as already reported by Yomo and by Paleg, but also certain components of the cytolytic system. The general pattern of pentose sugars and oligosaccharides liberated closely resembles that found when purified preparations of pentosanase are allowed to act on araboxylan for 50 hr., and the presence of cellobiose, laminaribiose and certain more complex β-linked glucose oligosaccharides is clear evidence for active hydrolysis by the β-glucanase system of enzymes. It will be realized that values given in Tables I–III for sugar release do not have any very precise meaning, since so many different types of sugars and oligosaccharides are involved: nevertheless, this liberation of reducing groups gave a clear measurable indication of carbohydrase activity in the tissue slices.

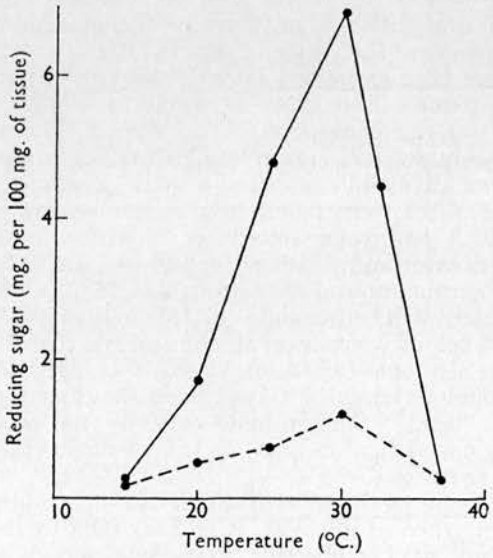

Fig. 1.—Release of reducing sugar (as apparent glucose) in 24 hr. at different temperatures. Continuous line—5 μg. of gibberellic acid per ml. of surrounding fluid; broken line—water.

Influence of temperature on sugar release.—The amounts of reducing sugar liberated from endosperm slices in 24 hr. at various temperatures are shown in Fig. 1. There is a well-marked optimum temperature for maximal activity at the surprisingly low figure of 30° C., and it is interesting to note that untreated endosperm slices show a similar

optimum temperature for sugar release, at a very much lower level.*

Enzyme Formation

Endo-β-glucanase and endopentosanase.— Although the results of the chromatographic analysis reported in Table IV strongly suggest that treatment with gibberellic acid causes liberation of active cytolytic enzymes, it seemed desirable to examine the treated endosperm slices and their exudates for presence of the relevant enzymes. The exudates were filtered and used directly without any attempts to purify the possible secreted enzymes; the tissue slices were first macerated in a *Micro-Wet-Grinder*, then extracted with 0·6% NaCl, and the clarified extracts were tested for presence of endo-glucanase and endopentosanase. Since the standard procedures of Preece *et al.*[17,18] were used to determine cytolytic activity, the values quoted in Table V are derived from measurements of the fall in viscosity of solutions either of pentosan or of β-glucan in presence of the various extracts. Results here have been expressed as activities per 100 g. of tissue. Two types of result are shown: in (*a*) the endosperm slices were cut from freshly-dehusked grain in which the embryos were alive and capable of growth, and in (*b*) the slices were taken from dehusked grain which had been stored for 3 weeks in a desiccator and which proved to be incapable of germination. Grains from sample (*b*) were tested with tetrazolium chloride and gave no red colour whatsoever in the embryo though the aleurone stained bright red: this material would certainly be classified by the maltster as "dead," though some capacity for biological reduction was still evident in the aleurone.

As far as the slices from "living" grain are concerned (Table Va), it is clear that treatment with gibberellic acid has caused a substantial enhancement (approx. 8-fold) in the total endoglucanase activity of the endosperm: endopentosanase, which could not be detected in the untreated slices, was produced under the influence of gibberellic acid, though none of the pentosanase was apparently secreted to the surrounding liquid. Slices from the apparently dead grain also responded to the presence of gibberellic acid, though results in this case were less spectacular, with the rise in glucanase activity to a little less than 5-fold. When storage was prolonged till the aleurone lost all capacity to reduce tetrazolium salts, there was no significant response to gibberellic acid.

TABLE V

ENZYME PRODUCTION* BY ENDOSPERM SLICES

(24 hr.; 25° C.)

Incubation medium	Endo-β-glucanase		Endopentosanase	
	Secreted	Retained in tissue	Secreted	Retained in tissue
(*a*) *Slices from grain with viable embryos*				
Water	0	66	0	0
Gibberellic acid	25	475	0	43
(*b*) *Slices from grain with dead embryos*†				
Water	0	60	0	0
Gibberellic acid	0	284	0	Trace

* Enzyme units: changes in reciprocal specific viscosity of standard substrate[17,18] expressed as values per 100 g. of tissue.
† But with aleurone staining with tetrazolium (see text).

Exoglucanase and exopentosanase. — Although simple untreated extracts can be used to estimate the activities of endo-enzymes, the presence of substantial amounts of sugars and oligosaccharides makes them inappropriate for determinations of exoglucanase or exopentosanase. The interfering sugars and oligosaccharides can certainly be removed by dialysis, but this process is rather slow and some inactivation of enzymes takes place before separation of carbohydrates is complete. However, a column of *Sephadex* (G 25, coarse) equilibrated and eluted with 0·6% NaCl successfully retained all sugars while allowing recovery of 80% of the original endo-β-glucanase activity; 100% recovery could be achieved at the expense of slight contamination with higher oligosaccharides. It was interesting to note that, when assays were being made of the endoglucanase retained in the endosperm slices, no activity could be recovered from the *Sephadex* column if water was used as equilibrant and eluent, suggesting that the enzyme is a globulin rather than an albumin.

When carbohydrate-free eluates—with 80% of the original endoglucanase still detectable—were tested for exoglucanase and exopentosanase, no real difference could be established between extracts from gibberellin-treated material and those from the controls: indeed, neither showed more than a

* Since this work was completed Paleg (*Plant Physiol.*, 1961, **36**, 829) has reported a similar temperature optimum for gibberellic acid-induced sugar release by endosperms of Prior barley.

trace of exo-enzyme activity. It is unlikely that these particular enzymes are retained on the column as firmly as are the oligosaccharides, and it therefore seems probable that neither exoglucanase nor exopentosanase is present in large amounts in the endosperm. Present evidence also suggests that these exo-enzymes are not markedly affected by gibberellic acid.

Effects of gibberellic acid and bromate acting jointly on endosperm slices.—Since one known effect of gibberellic acid on barley during malting is to increase proteolysis, it was of interest to determine what happened in endosperm slices in which proteolytic activity was largely suppressed. Potassium bromate and gibberellic acid were therefore added to endosperm slices, in the concentrations found by Macey & Stowell[12] to be optimal for suppressing proteolysis while allowing modification to proceed satisfactorily. In one set of experiments estimation was made of sugar release and also of nitrogenous compounds (determined by micro-kjeldahl) secreted to the ambient liquid and, in a second series, β-glucanase was estimated both in the medium and in the tissue slices; typical results of these sets of determinations are given in Table VI. It is clear that gibberellic acid does induce formation of soluble nitrogenous material and that the presence of bromate has suppressed the release of the nitrogenous compounds to the medium, while still allowing considerable secretion of reducing sugar. It may be noted that, in determinations where both gibberellic acid and bromate were present, the amounts of sugar found in the medium were substantially less than those recorded in presence of gibberellic acid alone. Endo-β-glucanase formation, however, was at least as extensive in presence of gibberellic acid + bromate as it was with gibberellic acid alone: the partition of this activity between medium and tissue was very variable and only the total (excreted glucanase + tissue glucanase) is reported in Table VI.

TABLE VI

EFFECT OF GIBBERELLIC ACID AND POTASSIUM BROMATE ON ENDOSPERM SLICES

	Incubation medium (24 hr. at 25° C.)		
	Water	Gibberellic acid	Gibberellic acid + bromate
Sugar release*	0·5	3·1	2·7
Nitrogen release*	0·08	0·23	0·08
Total endo-β-glucanase†	99	436	516

* mg. per 100 mg. tissue. † Units as in Table V.

Effect of gibberellic acid on barley embryos.—Although the present work has been concerned mainly with the effects of gibberellic acid on endosperm, some study has also been made of possible interactions with barley embryos. Grain was steeped for 2 hr. and the embryos excised therefrom were grown for 26 hr. at 25° C. either on water or on gibberellic acid solutions. After maceration of the seedlings and extraction in 0·6% NaCl, the filtered solutions were analysed for exo- and endo-glucanase and for pentosanase activities. From a knowledge of the 1000-corn weight of the grain used, it is possible to calculate the approximate contribution to enzyme activity made by embryos from 100 g. of whole barley. These figures are given in Table VII, where it can be seen: (*a*) that the contribution made by the embryo to endoglucanase activity of the whole corn is very roughly of the same order as that made by the endosperm before treatment with gibberellic acid; and (*b*) that gibberellic acid does not augment the endo-β-glucanase content of the embryo.

TABLE VII

EFFECT OF GIBBERELLIC ACID ON ENDO-β-GLUCANASE OF EXCISED EMBRYOS

(Growth conditions: 26 hr. at 25° C. in the dark)

	Incubation medium	
	Water	Gibberellic acid
Endo-β-glucanase* secreted	0	0
Endo-β-glucanase in tissue	149	142

* Enzyme units are expressed as values for 100 g. intact grains.

Considerable reducing group activity was generated when embryo extracts were incubated with β-glucan, but calculation showed that all of it could be ascribed to the effects of endo-β-glucanase, and neither with the control nor with the gibberellic acid-treated preparations was there any evidence

for additional exo-enzyme activity in the embryo.

No cytolytic enzymes appeared to be excreted from the embryo into the surrounding fluid, and addition of liquid in which embryos had been grown, either for 24 hr. or for 48 hr., had no measurable effect on sugar release or on glucanase formation in endosperm slices. However, when excised embryos were maintained in the same petri dish as endosperm slices, though not in contact with them, there was a definite development of endo-β-glucanase in the separated slices. These observations suggest that the presence of endosperm may stimulate the embryo to produce material which in turn causes the liberation of β-glucanases from the aleurone, and this interaction of embryo and endosperm is being more fully investigated.

Action of gibberellic acid on aleurone extracts.—In order to determine more precisely the point at which gibberellic acid acts on endo-β-glucanase, additions of gibberellic acid were made at various stages during preparation and purification of the enzyme. No change in enzyme activity could be detected when gibberellic acid was added to mixtures of enzyme and substrate, either immediately before the assay was made or during the course of the determination. Again, when extracts were prepared from untreated endosperm slices and gibberellic acid was added to the filtered extracts, which were then incubated overnight, no enhancement of activity could be detected. However, when dry aleurone cells from ungerminated grain were slurried in 0·6% NaCl, ground finely in a glass mortar and then exposed to 1 μg. of gibberellic acid, substantial amounts of endo-β-glucanase were released into the salt solution, though none could be detected in the controls which were incubated without gibberellic acid. It seems, then, that gibberellic acid will exert its influence either on intact aleurone cells or on homogenates prepared in saline, but will not act on solubilized protein, separated from the cells.

Discussion

From the results presented above, it is abundantly clear that gibberellic acid can stimulate secretion of hydrolytic enzymes—proteinase, amylase, endo-β-glucanase and pentosanase—in fragments of barley endosperm which have been separated from their embryos. Although the precise nature of the biochemical mechanisms involved remains obscure, one effect of gibberellic acid, *viz.* induction of increased proteolytic action, can be suppressed without significantly affecting the increase in carbohydrase activity. It would therefore appear that the primary effect of gibberellic acid is not an action on proteinase allowing this, in turn, by its action on occluding deposits of nitrogenous material, to expose both carbohydrates and carbohydrases and so allow rapid degradation of cell wall material. The large-scale experiments carried out by Macey & Stowell[12] have shown that this dissociation of active proteolysis from accelerated carbohydrate modification is also possible in the more complex conditions prevailing during malting, and, as will be discussed later, this separation of two biochemical effects does give some indication of the locus in the cell at which gibberellic acid is exerting its influence.

The surprisingly low temperature optimum (30° C.) for the action of gibberellic acid on endosperm slices strongly suggests that the fundamental effect is not a simple enzyme activation. All the hydrolytic enzymes under consideration at present are capable of intense activity, *in vitro*, at temperatures well above 37° C. Moreover, when endosperm slices were maintained at 37° C. in presence of gibberellic acid for periods of up to 48 hr. and then transferred to 25° C., the range of effects associated with gibberellic acid treatment—enhanced enzyme activity and sugar secretion—took place in the usual manner. Apparently, then, no permanent damage was caused by the higher temperature, but rather a temporary inhibition of the characteristic effects of gibberellic acid. Whether this inhibition is due to a change in the properties of gibberellic acid itself (*e.g.* a reversible epimerization which is strongly temperature dependent) or whether some unspecified cell component is in some way modified at temperatures above 30° C., thus protecting it from the action of gibberellic acid, cannot as yet be decided, but it is worth noting that some of the effects of coumarin (also a lactone and usually regarded as a growth inhibitor) are likewise diminished at temperatures of above 30° C.[4] Coumarin, incidentally, inhibits many of the hydrolytic enzymes of wheat,[16] and a study of the joint action of coumarin and gibberellic acid on barley endosperm would be well worth while.

This peculiar effect of temperature on the activity of gibberellic acid is paralleled by the behaviour of plants of temperate regions grown at different temperatures. Thus, if barley is grown for 4 days at temperatures ranging from 15° to 37° C. and the total root production (*i.e.* the sum of the lengths of all rootlets produced) is plotted against temperature, a curve is obtained which is virtually superposable on that shown in Fig. 1. This does not imply any necessary connection between root growth and gibberellin action, but it does suggest that the effects of gibberellic acid may fit naturally into the complex pattern of hormone-mediated plant growth, differently affected at different temperatures, and that response to gibberellin thus constitutes one aspect of a much wider problem in plant physiology.

The clear demonstration (Table II) of the importance of the aleurone layer in the reaction with gibberellic acid is of interest not only in relation to the effects of exogenously supplied gibberellic acid on barley during malting, but also in relation to events which occur when barley germinates without additions. These aleurone cells have attracted the attention of maltsters and of botanists for very many years, and before any assessment is made of the effects of gibberellic acid on aleurone, it is desirable to survey conclusions reached by earlier workers regarding the part played by aleurone in cereal germination.

Barley aleurone.—One of the first significant studies of barley aleurone was that made by Brown & Escombe in 1898.[2] Working with fragments of endosperm containing both aleurone and starch cells, and with these fragments floated on mica rafts to allow free diffusion of products of degradation, Brown & Escombe were able to show that digestion of cell walls of the starchy endosperm took place slowly from the aleurone layer down through the underlying starchy cells: bacterial attack was not responsible for this cytolysis, since micro-organisms, if present, invariably manifested their activities initially at the exposed starchy end of the fragment. Again, when embryos were grafted on to untreated endosperms, these embryos grew well and the supporting endosperms were digested, but when living embryos were grafted on to endosperms which had been "killed" by 24-hr. immersion in chloroform-water, growth was poor and modification was largely restricted to the region of the endosperm abutting on the scutellum. From the results of many such experiments, Brown & Escombe concluded that barley aleurone was largely implicated in cell-wall digestion in the endosperm; they further noted, however, that the aleurone-mediated cytohydrolysis took place much more rapidly when the embryo was present than when it had been removed. This important work thus provided the first suggestion of some influence of embryo on aleurone, so causing enhancement of an existing potentiality for enzyme secretion.

Although not all subsequent workers supported Brown & Escombe,—Mann & Harlan,[13] *e.g.*, profoundly disagreed with their findings—the results of a detailed study of grass aleurone carried out by Schander[22] have fully confirmed the suggestion that aleurone cells are of very considerable importance in normal germination. Schander found that, with rice and *Stipa*, prior removal of aleurone from dry grains completely prevented seedling growth when the seeds were subsequently given optimum conditions for germination; with barley the effect of polishing was to cause a decline in acrospire length from 9 cm. in 14 days (untreated) to 2 cm. (polished). In general, cereals with a large volume of aleurone relative to scutellum did not grow at all after removal of the aleurone, though those with a large scutellum did accomplish some growth. Additions of glucose, auxin, or aleurone extract did not allow growth of polished rice; the only occasions on which aleurone-free rice grew satisfactorily were those when contamination by fungi (of unspecified nature) had occurred. Removal of a ring of aleurone directly above the scutellum had the same effect as complete polishing, and, to permit growth and endosperm modification, contact of aleurone and scutellum had to be maintained.

Schander also removed embryos from unsteeped barley by grinding off the overlying endosperm. These embryos formed no rootlets and produced acrospires only a few mm. long; on the other hand, embryos excised from steeped grain grew well. It will be realized that, in almost all other work reported with excised embryos, excision has been performed after the grain has been steeped for 2 hr., since it is technically difficult to remove the embryo from an unimbibed corn. It has been tacitly assumed that brief contact with water exercises no

important effect on the grain but, as Schander's work shows, such an assumption may be ill-founded.

From the results of his observations Schander concluded that, when rice seeds germinate, two important transfers of material take place as soon as the grain is wetted: one substance (X) is transported from germ to aleurone via the portion of the scutellum nearest to the acrospire, and a second substance (Y) is moved from aleurone to embryo, this time via the rootlet end of the embryo. X is implicated in changes involved in endosperm modification and Y is a growth factor which is essential for seedling growth and which is not normally present in the embryos of mature dry seeds.

Gibberellic acid in barley germination.—As would be expected from the results presented in the Experimental section, Schander's postulated substance X can be replaced by gibberellic acid exogenously applied to grains which have been ringed above the embryos to deprive them of contact with aleurone. Full details of this aspect of the effects of gibberellic acid will be published later, but it may be noted in passing that, although gibberellic acid caused endosperm modification to proceed in ringed grain (though not in grain which had been wholly deprived of aleurone), it did not stimulate seedling growth. Although we have no rigid proof that gibberellic acid is implicated in the normal modification of untreated barley, it seems highly probable that changes in the endosperm are initiated by a mechanism involving translocation of material—with a physiological action very similar to that of gibberellic acid—from embryo to aleurone. This material may indeed be the endogenous barley gibberellin which has been studied by Radley[19] and by Lazar *et al.*[11]

Although gibberellic acid had a marked effect on the endoglucanase of the aleurone (Table V), it did not appear to cause enhancement of endoglucanase in the excised embryo (Table VII). Now, if the endogenous gibberellin of barley is restricted to the embryo in ungerminated grain, two possible situations can be envisaged: either the gibberellin has exerted its influence on the hydrolytic enzymes of the resting embryo during maturation so that the full potential of hydrolase activity is released as soon as the embryo is extracted with saline, or, alternatively, the process of grinding brings gibberellin into contact with inert hydrolytic systems, so causing their activation. The first possibility would be expected if gibberellin and enzymes are free in the cell and the second if either component is bound in some manner: either condition would allow full expression of hydrolytic potential when the embryos were ground and extracted.

Suggested mode of action of gibberellic acid.—As has been reported above, gibberellic acid can exert its effect either on intact aleurone cells or on homogenates, but it is apparently without effect on extracts. Secondly, gibberellic acid shows its most pronounced effects on hydrolytic enzymes. If these two observations are taken together, it can be deduced that gibberellic acid must act on particulate hydrolytic enzymes or on assemblies of hydrolytic enzymes. The obvious candidate for this assembly of enzymes is the lysosome of de Duve *et al.*[5] These organelles, as represented in liver cells, are somewhat lighter than mitochondria. After differential centrifugation lyosomes can be induced to liberate their associated hydrolytic enzymes by treatment with lipase or with pepsin, suggesting that their membranes are of lipoprotein and their contents are chiefly hydrolases. Little is known about analogous structures in plant cells, but it does seem that interaction between gibberellin and lysosome-like organelles can best explain the explosive release of α-amylase, proteinase and β-glucanase which is regularly observed, not only when gibberellic acid is added to barley, but also when barley germinates without exogenously-added supplies of gibberellin. Briefly, then, it is suggested that when barley grain germinates, gibberellin migrates from the embryo along the aleurone, catalysing release of hydrolytic enzymes from lyosomes; these hydrolytic enzymes then act, independently of gibberellin, to degrade the appropriate substrates. Addition of suitable inhibitors, such as bromate, can influence the activity of different members of the hydrolytic complex, only after they have been released by gibberellin. It must be emphasized that this is a hypothesis only, but it does seem to accommodate the facts known about gibberellic acid and barley germination. It is, incidentally, of interest that reports have occasionally been made of particles which resemble mitochondria or aleurone grains, and which appear to migrate from the living parts of cereal seeds into the starch cells.[8]

These particles would be well worth investigating by modern techniques.

The aleurone and the embryo in relation to malting.—The experimental results and the hypothesis presented above suggest explanations of a number of observations which have been made on the behaviour of barley during malting. Thus, the gradual advance of cell wall hydrolysis from the embryo to the tip of the grain, slowly in the centre of the grain and rapidly in the sub-aleurone layer, follows the course which would be expected if release of endo-β-glucanase is a response to gibberellin migrating through the aleurone. The defective modification of cell walls which is not infrequently observed near the furrow can be correlated with the fact that in the furrow the aleurone cells are somewhat disorganized and are only one layer deep instead of the usual three: the total amount of hydrolases available for release near the chalaza would thus be less than that in other parts of the grain. Again, there are records of grain which has failed to grow but which in presence of gibberellic acid has given well-modified malt of high extract value;[12] this situation is analogous to events reported in Table Vb, where endosperm slices cut from grain with dead embryos still exhibited a marked response to gibberellic acid. In this connection it is relevant to enquire how the added gibberellic acid penetrated the grain: little seems to be known about the means of entry of gibberellic acid, though it is widely believed that entry is possible only after chitting has begun.

It may be as well at this point to give a warning: although, with the aid of added gibberellic acid we have been able to make material which analytically and organoleptically resembles malt from barley in which the embryos have been killed, this has been achieved only with grain in which the aleurone, as judged by response to tetrazolium chloride, was capable of respiring. The dehusked samples of grain retained viability for 3–4 weeks in a desiccator, but, as storage was prolonged, the magnitude of the response to gibberellic acid diminished until, eventually, even the aleurone showed no reaction to added gibberellic acid. The cause of this rather rapid loss of viability by dehusked grain is not known, but it has also been reported with other grass seeds.[22] Thus, although the aleurone is apparently more robust than the embryo (see, *e.g.* Brown & Escombe[2]) and instances may occur in commercial practice when pregerminated grain with dead rootlets can modify in response to gibberellic acid acting on a "living" aleurone, it is certainly not claimed that malt can be made from completely dead grain.

Nor is it suggested that the embryo, and especially the scutellum, plays no part in endosperm degradation other than supplying to the aleurone material with properties resembling those of gibberellic acid. There is no doubt that the scutellum can secrete α-amylase, and the presence of endo-β-glucanase inside the embryo is also certain. The failure to detect secretion of endo-β-glucanase by cultured embryos may be analogous to the similar absence of secreted α-amylase from embryo cultures which are grown in presence of assimilable sugars,[3] and events taking place in isolated embryos may differ widely from metabolism of embryos which are in close contact with the hemicelluloses of adjacent endosperm cells.

Nevertheless, all the available evidence suggests that the aleurone of barley is of outstanding importance in barley germination, and, though the relationships between gibberellins, hydrolytic enzymes, organelles like lysosomes, and respiratory activity in the aleurone cells remain obscure, we hope to investigate them further in the near future.

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ULTRA-STRUCTURE OF CARYOPSES OF THE GRAMINEAE

I. ALEURONE AND CENTRAL ENDOSPERM OF *BROMUS* AND BARLEY

BY

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I. ALEURONE AND CENTRAL ENDOSPERM OF *BROMUS* AND BARLEY

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Sections of ungerminated caryopses of barley and of *Bromus* examined by electron microscopy are found to contain in the cells of the aleurone layer the usual membranes and cell organelles; additionally there are present vacuoles with (in barley only) characteristic electron-dense inclusions. Large numbers of unidentified bodies, 0·1–0·4 μ. in diameter, are also found in the aleurones of both species. The aleurone cell walls have a fibrillar cellulose-like structure; the walls of the central endosperm are less highly organized. Starch grains and deposits of material of medium electron density, probably proteinaceous in nature, are the only observed inclusions in the central endosperm cells.

Introduction

Results of recent biochemical studies[1,8] have focused attention on the properties of the aleurone layer of cereal grains. This tissue is now known to be implicated in the liberation of certain of the hydrolytic enzymes which participate in modification of the reserve material of the central endosperm; it is also the region of the grain which responds to addition of gibberellic acid. Calculation suggests that more than 80% of the total α-amylase[1] and endo-β-glucanase[7] of barley malt is derived from the activities of the aleurone cells and, though comparable figures are not available for proteinase and endopentosanase, it is clear that these enzymes also can be produced in substantial amounts by the aleurone.[8] The recent demonstration[5] that gibberellic acid is present in ripening barley grains has given added weight to the hypothesis that, during germination, endogenous gibberellic acid interacts with the aleurone to catalyse the mobilization of hydrolytic enzymes. Although the biochemistry of the aleurone-gibberellin interaction is not yet fully understood, there can be no doubt at all about the importance of the aleurone in the metabolism of germinating barley.

Other grass seeds—wheat,[3] oats[12] and certain species of *Bromus*[6]—respond to additions of gibberellic acid in a manner generally similar to that observed with barley and, in *Bromus* at least, it is also the aleurone layer of the seed which responds to the hormone. To supplement the biochemical studies of

the effects of gibberellic acid on barley, a survey is now being made of the fine structure of the aleurone and starchy endosperm cells of barley and of *Bromus* during germination without additives and in endosperm slices treated with gibberellic acid. *Bromus* has been chosen for study in conjunction with barley because, although this grass is phylogenetically distinct from the Hordeae, it resembles barley rather closely in the general pattern of evolution of cytolytic enzymes during germination. It is dangerously easy to mistake artefacts for reality in interpreting electron micrographs and it is hoped that, by examining two apparently unrelated species of the Gramineae, this danger may be minimized.

The present Communication is a record of observations made on the ultra-structure of endosperm prior to germination; changes taking place in aleurone and in starchy endosperm in response to germination and in response to addition of gibberellic acid will be reported later.

Technique

The barley used was Proctor from the 1963 harvest. Slices of endosperm, 2 mm. thick, were cut from dehusked grain, halved through the furrow and steeped in water at 25° C. overnight. These endosperm fragments were then ground gently in a glass homogenizer until all the starchy tissue was suspended in the distilled water used as grinding medium. The aleurone was separated by passing the mixture through a sieve and washing the residue thoroughly: the isolated aleurone was then processed in the manner described below. Preparations were also made from slices containing both aleurone and starchy endosperm; these gave electron micrographs which were similar to those from the isolated aleurone, and it thus appears that the separated aleurone is essentially unchanged in its ultrastructure.

For *Bromus*, caryopses of *B. inermis* Leyss were freed from husk by treatment with 50% sulphuric acid, washed and stored moist at 25° C. overnight. 1-mm. slices were cut transversely from the endosperm and smaller (approximately 0·5-mm.) fragments were cut from these slices to include both aleurone and starchy endosperm. It will be realized that significant changes may have taken place during the 18-hr. equilibration of the grain, or grain fragment, with water, but it has as yet proved technically impossible to prepare electron micrographs from unimbibed material.

Processing.—The isolated aleurone, or the endosperm fragments, were normally fixed at 5° C. in 0·6% buffered potassium permanganate over a range of times extending from 30 min. to 4 hr. The fixed material was dehydrated in increasing concentrations of ethanol and embedded under reduced pressure in methacrylate (88% butyl methacrylate + 12% methyl methacrylate polymerized at 60° C. in presence of 1% dichlorobenzoyl peroxide). The polymerized blocks were trimmed and sections were cut on to 15% ethanol by a *Cambridge* ultramicrotome. The sections were usually of 500 Å thickness, though on occasions sections of 250 Å were examined. Any ripples present in the sections were evened out by treatment with xylol vapour and the sections were mounted on uncoated grids and subsequently coated with a thin layer of carbon on one side only. The specimens were examined and photographed in an *Akashi* TRS 50 E1 electron microscope fitted with a specially modified D.C. filament and a 50 kv. E.H.T.

Explanation of Figures

Fig. 1.—Aleurone of *Bromus*. (*a*) fixation in unbuffered $KMnO_4$; (*b*) pre-fixation in neutral formalin followed by fixation in buffered $KMnO_4$; (*c*) as in (*b*).

Fig. 2.—Aleurone of *Bromus*. (*a*) fixation in unbuffered $KMnO_4$; (*b*) prefixation in neutral formalin followed by fixation in buffered $KMnO_4$; (*c*) as in (*b*); (*d*) prolonged fixation (4 hr.) in buffered $KMnO_4$.

Fig. 3.—Barley aleurone. (*a*), (*b*) and (*c*) fixed in buffered $KMnO_4$.

Fig. 4.—Starchy endosperm of *Bromus*. (*a*) fixation in unbuffered $KMnO_4$; (*b*) prefixation; (*c*) fixation in unbuffered $KMnO_4$.

(D.B.—dense body; E.R.—endoplasmic reticulum; I—inclusions, mainly vacuolar; I.S.—intercellular substance; M—mitochondrion; M.L.—Middle lamella; N—nucleus; N.M.—nuclear membrane; P—plastid; Pr—medium density deposit, probably protein; S—starch grain; S.M.—membrane surrounding "protein" sac; U.B.—unidentified body; V—vacuole; V.M. vacuolar membrane; W—wall (in general); W_{1-3}—three apparent layers of endosperm wall.

Observations

Internal Structure of the Aleurone Cell

The general structure of a typical aleurone cell from *Bromus* is shown in Fig. 1. In addition to structures which are normally found in undifferentiated plant cells—*e.g.*, nucleus, mitochondria, plastids, endoplasmic reticulum and plasmalemma—there are also small vacuoles which here are very distinct indeed, and which are enclosed in sharp and well-preserved membranes. Other less familiar inclusions are also present and not all these organelles can as yet be identified with certainty. However, the descriptions which follow are based on results obtained from a large number of different preparations, and we are reasonably confident of the authenticity of these unfamiliar inclusions.

Nucleus.—The nuclei are from 15 to 20 μ. in diameter and on occasions the presence of nucleoli has been demonstrated. The nuclear envelope is distinctly a double layer: the outer membrane passes into the cytoplasm and is apparently continuous with the endoplasmic reticulum (Fig. 1 *a*). Holes, about 500–800 Å in size, are visible in the inner membrane as indicated by arrows in Fig. 1 *a*.

Mitochondria.—In *Bromus*, membrane-enclosed organelles (M) of the mitochondrial type, 0·7–1·4 μ. in diameter, can be seen in aleurone cells and a close examination shows that they are of the same internal structure as those reported from most other plant cells. The number of characteristic, easily-recognized mitochondria present seems to depend on the developmental stage of the cell, and many of the cells of *Bromus* and most of the barley aleurone cells (at the stages under discussion) contained no typical mitochondrial structures. It is possible that there is some connection between the appearance of mitochondria and the small vacuoles (V) which are often present in fairly large numbers (Fig. 2 *b*). This possibility will be considered more fully later.

Plastids.—Structures resembling plastids (P) have been detected in some aleurone cells (Fig. 1 *a*), but it is possible that these are simply mitochondria of the type reported by Hrsel *et al.*[4] to be present in the scutellum of wheat.

Vacuoles and vacuolar inclusions.—In all specimens of *Bromus* and of barley examined (Fig. 1; Fig. 2, *a* and *b*; Fig. 3), several small, regularly-shaped vacuoles (V) are found. These vacuoles are bounded by a distinct membrane and they are usually associated with regions of medium-electron-density material which is probably proteinaceous in nature. In *Bromus* this presumptive protein (Pr) is also enclosed in a membrane which is apparently continuous with the endoplasmic reticulum (Fig. 1 *a*), but in barley (Fig. 3, *a*, *b* and *c*) no evidence of such membranes has been found. It is possible that this medium-electron-density deposit may be a region of protein synthesis, especially where it is associated with the endoplasmic reticulum.

From Fig. 1, *a* and *c*, it seems probably that the vacuoles increase in number by a "budding" process which results in the formation of a large number of smaller vacuoles (Fig. 2 *b*). Examination of Fig. 2 *b* shows that there is often a considerable number of these smaller vacuoles present within one cell. It may be suggested that these "newly-budded" vacuoles are associated with respiration, in analogy with observations recently made by Polakis *et al.*[10] for yeast.

In all the specimens of *Bromus* examined, most of the vacuoles contained only an almost electron-translucent matrix (Fig. 1 *a*) whereas barley (Fig. 3) contained additionally many vacuoles with almost spherical electron-dense inclusions (I). There appears to be a gradation in the electron density of these vacuolar inclusions (I, 1–3 in Fig. 3 *a*). The significance of these inclusions of varying density is unknown: a gradation in development could be inferred, but there is as yet no evidence to indicate the direction of any possible trend. Similar vacuolar inclusions have been noted in wheat embryos[11] and in the aleurone of ripening wheat.[2] Buttrose[2] has provided convincing evidence for linking these vacuolar inclusions with the "aleurone grain" familiar from optical microscopy.

Unidentified bodies.—These small structures, 0·1–0·4 μ. in diameter and labelled U.B. are present in both barley and *Bromus*. In barley they are regular structures, 0·3–0·4 μ., and are electron-translucent. In *Bromus* they are smaller than 0·3 μ. and appear to be electron-dense (Fig. 1 *a*), though in other respects they are similar to those of barley, being vesicular in structure and provided with a definite limiting membrane (Fig. 2 *c*). In both *Bromus* and barley, these unidentified bodies are associated with the medium-density deposits (Pr) shown in

Fig. 1 *a* and in Fig. 3 *c*. They are also concentrated round the periphery of the cell, adjoining the plasmalemma (Fig. 1, *b* and *c*).

The differences in electron-density do not appear to be directly due to hydration, as was suggested by Buttrose,[2] since the U.B. remain electron-translucent in isolated barley aleurone which has been soaked in water for up to 24 hr., without influence from the embryo.

Dense bodies.—These small inclusions (D.B.) are visible only in the aleurone cells of barley (Fig. 3 *c*). They are widely dispersed throughout the cytoplasm, but they do not regularly appear to be associated with the U.B. Their significance is unknown, but similar structures have been detected in cells of cereal embryos.[11]

Internal Structure of the Starchy Endosperm Cell

This part of the investigation is still in an initial stage: indeed, little of interest can be expected until the starch-filled cells come under the influence of hydrolytic enzymes from the aleurone. However, Fig. 4 (*a* and *b*) shows the typical starch granules together with membrane-enclosed deposits of medium-density material which is probably proteinaceous in nature. Fine detail of the cell structure is lacking and no organelles resembling mitochondria have been detected. As would be expected from results with optical microscopy, which indicate only occasional remains of degenerating nuclear material, no evidence was found for the presence of a nucleus. The starchy-endosperm cells adjoining the aleurone layer contain a large number of small (less than 1·5 μ.) starch grains as well as the normal (1·5–4 μ.) grains, whereas few of the smaller grains are found in the inner tissue of the caryopsis.

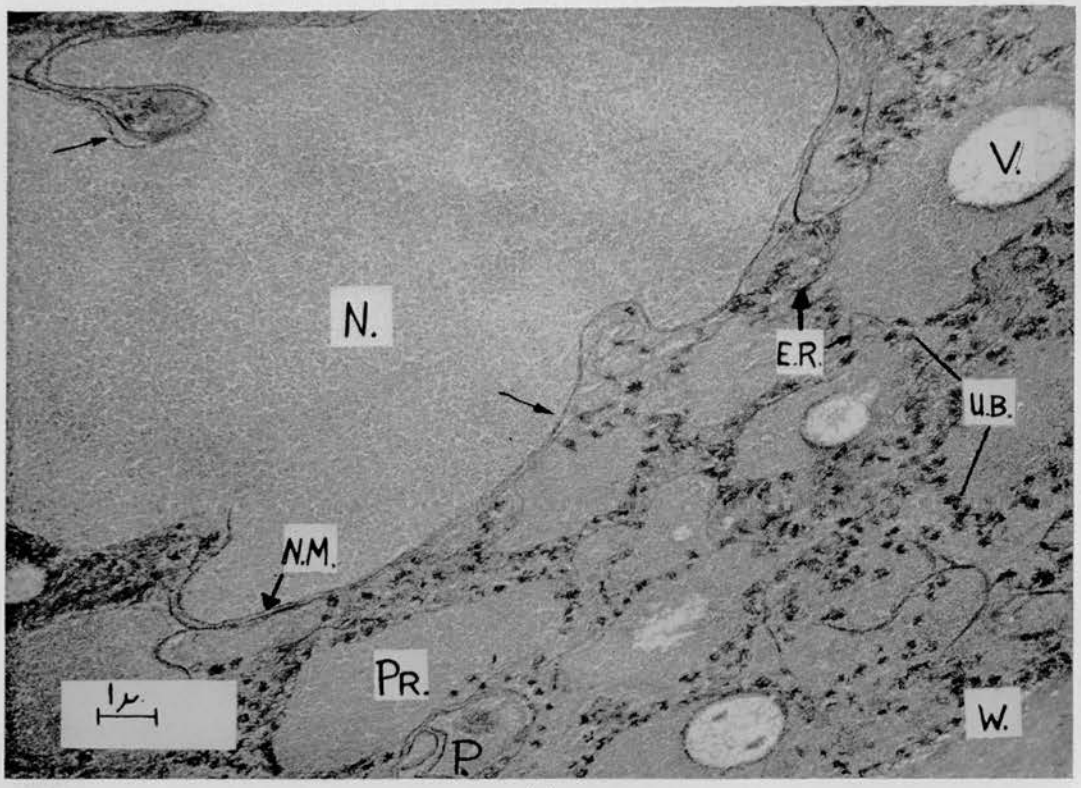
Comparison of Cell-wall Structure in Aleurone and in Starchy Endosperm

Aleurone wall.—The structure of a typical aleurone cell wall can be seen in Fig. 1, *b* and *c*. The wall has a fibrillar structure similar to that seen in most normal plant cells, and, taking its appearance in conjunction with results of earlier biochemical studies, it is reasonable to attribute this largely to cellulose. The fibrillar structure can be clearly demonstrated by prolonged treatment of the tissue with potassium permanganate, which results in oxidation of interfibrillar material and simultaneously induces separation of the resistant fibres (Fig. 2 *d*). The presence of a definite middle lamellar region has also been demonstrated in the aleurone.

Starchy-endosperm wall.—In Fig. 4, *a*, *b* and *c*, it can be seen that the endosperm wall of *Bromus* is exceedingly thick, though it has a rather less well-defined structure than that of the aleurone. Nevertheless, there is a considerable degree of organization, possibly fibrillar, still present. The distribution of cellulose in the caryopsis of *Bromus* is not fully known, though preliminary studies indicate that true cellulose is not present in large amounts in the wall of the starchy endosperm cell: the organized material, which is birefringent, may therefore be hemicellulose, which is certainly present in high concentrations in *Bromus*. Close examination of the micrographs (Fig. 4 *c*) suggests that this wall is in fact made of more than one distinct layer: there is a well-defined outer region within which can be seen one or possibly two more amorphous layers, the innermost of which appears to extend into the lumen of the cell as a form of matrix round the starch granules and other inclusions. Eventually, it should prove possible to relate these morphological observations to earlier biochemical[9] work in which cell-wall material has been fractionated into water-soluble, alkali-soluble and encrusting hemicellulose.

Discussion

Cereal grains and grass seeds are rather refractory objects as regards processing for electron microscopy, and the methods used (permanganate fixation followed by embedding in methacrylate) can lead to implosion of vesicles and formation of various types of artefact: these methods, however, are the only ones which, in our hands, have given consistently successful preparations. Despite the use of permanganate and methacrylate, membranes have been well preserved (Figs. 1–4) and the familiar subcellular organelles present a normal appearance: it is therefore reasonable to assume that the unfamiliar unidentified bodies are also authentic cell components.



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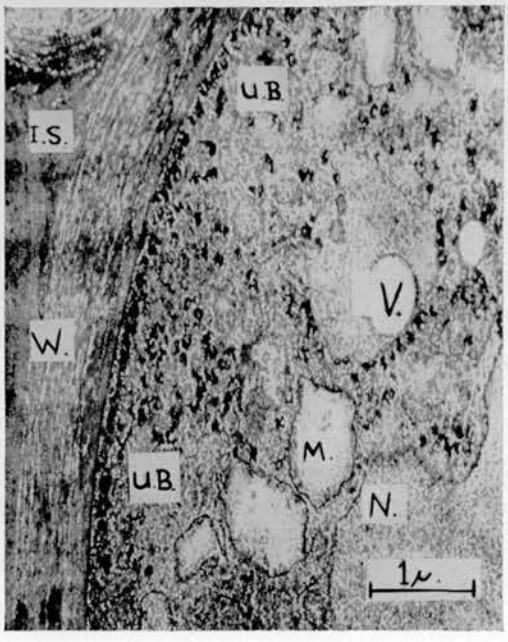

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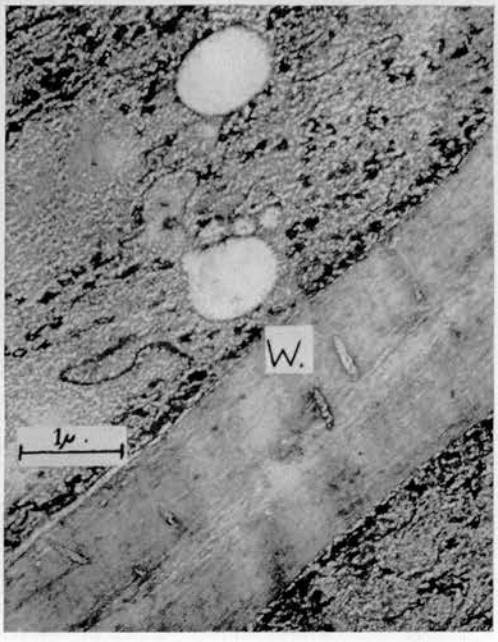

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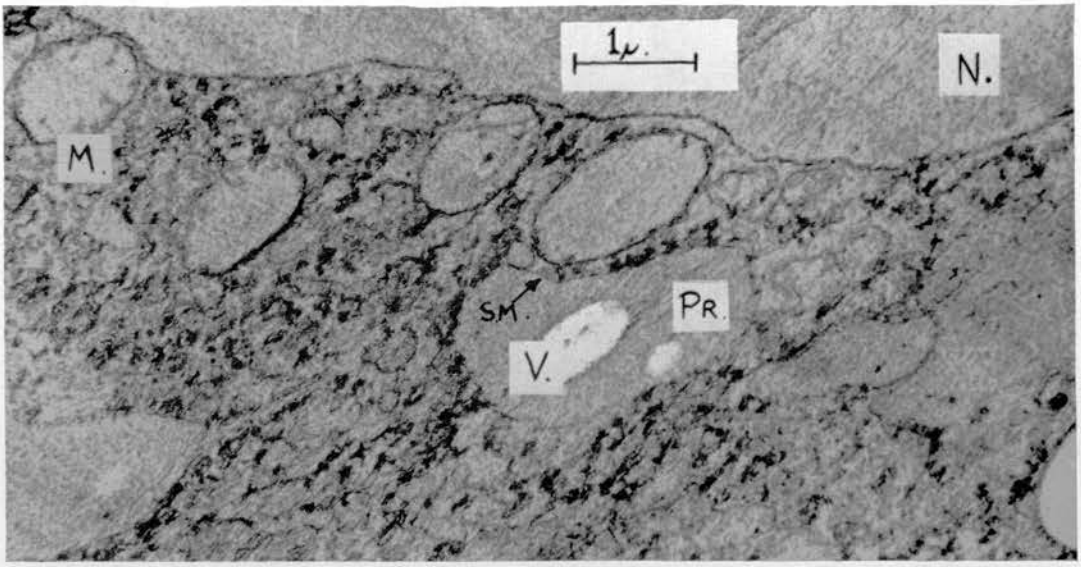
Fig. 1.—Aleurone of *Bromus*.



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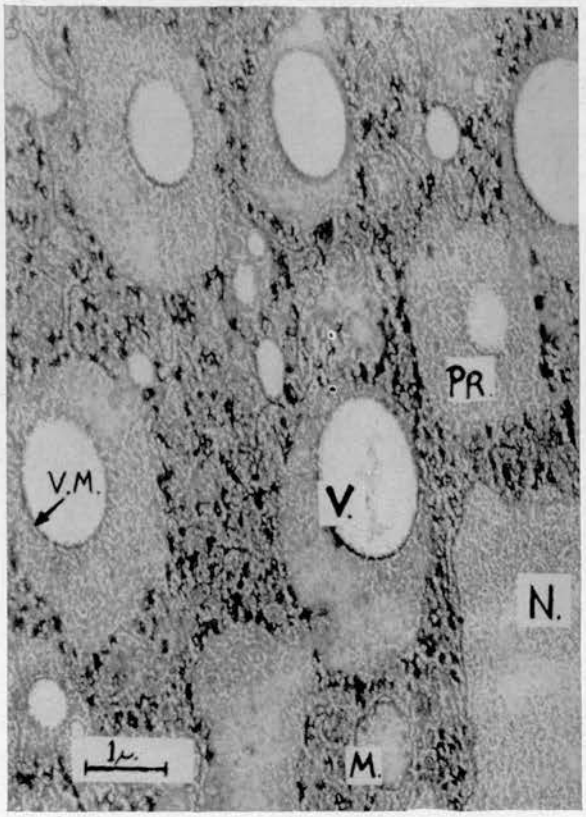

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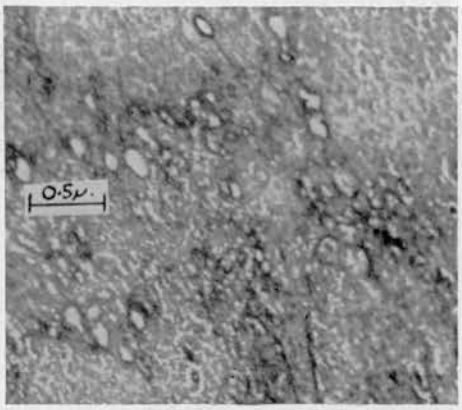

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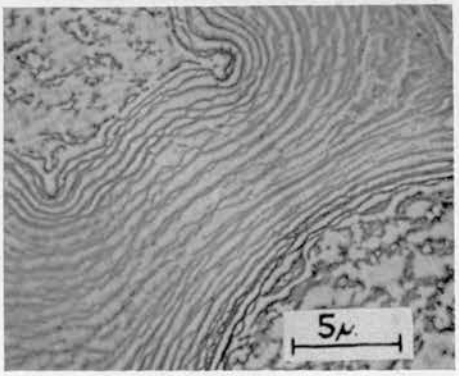

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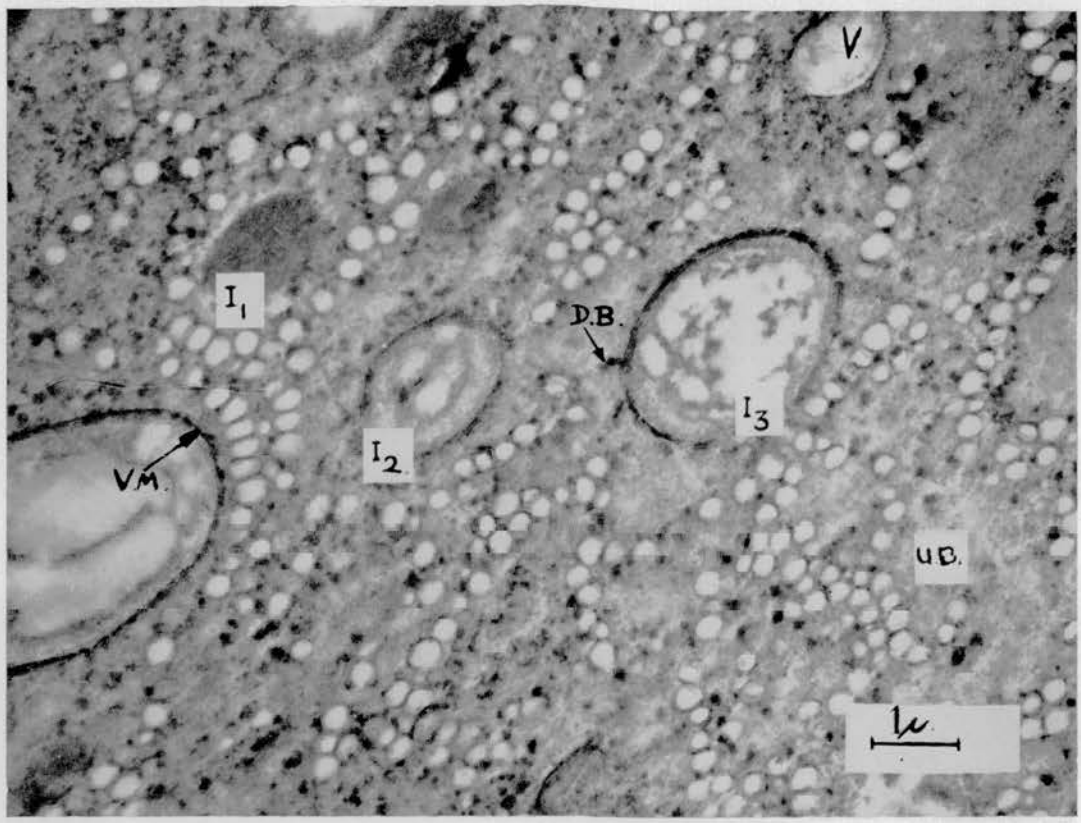
Fig. 2.—Aleurone of *Bromus*.



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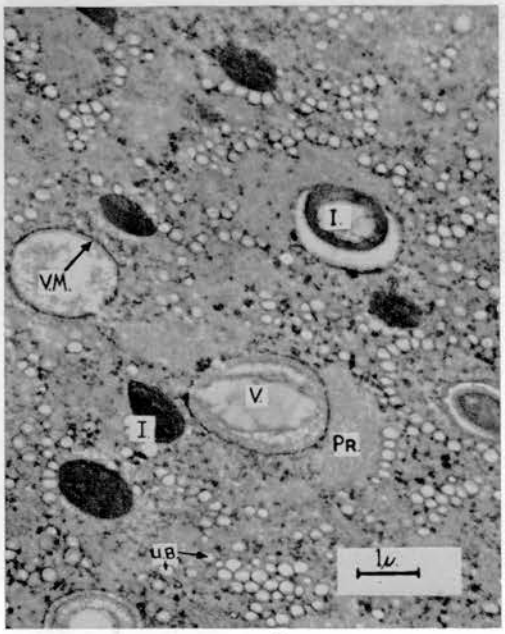

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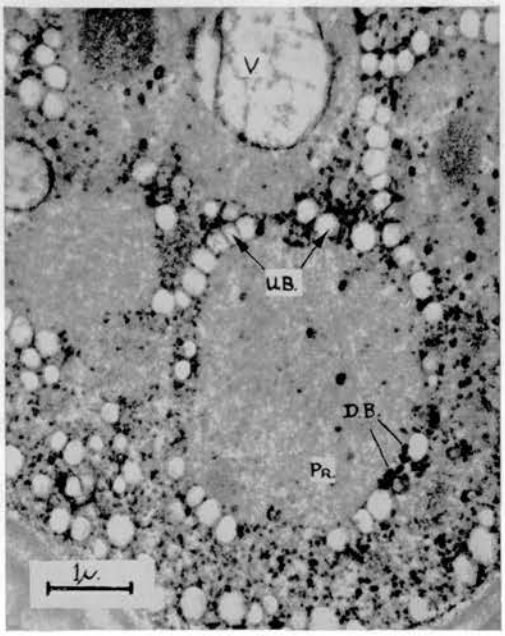

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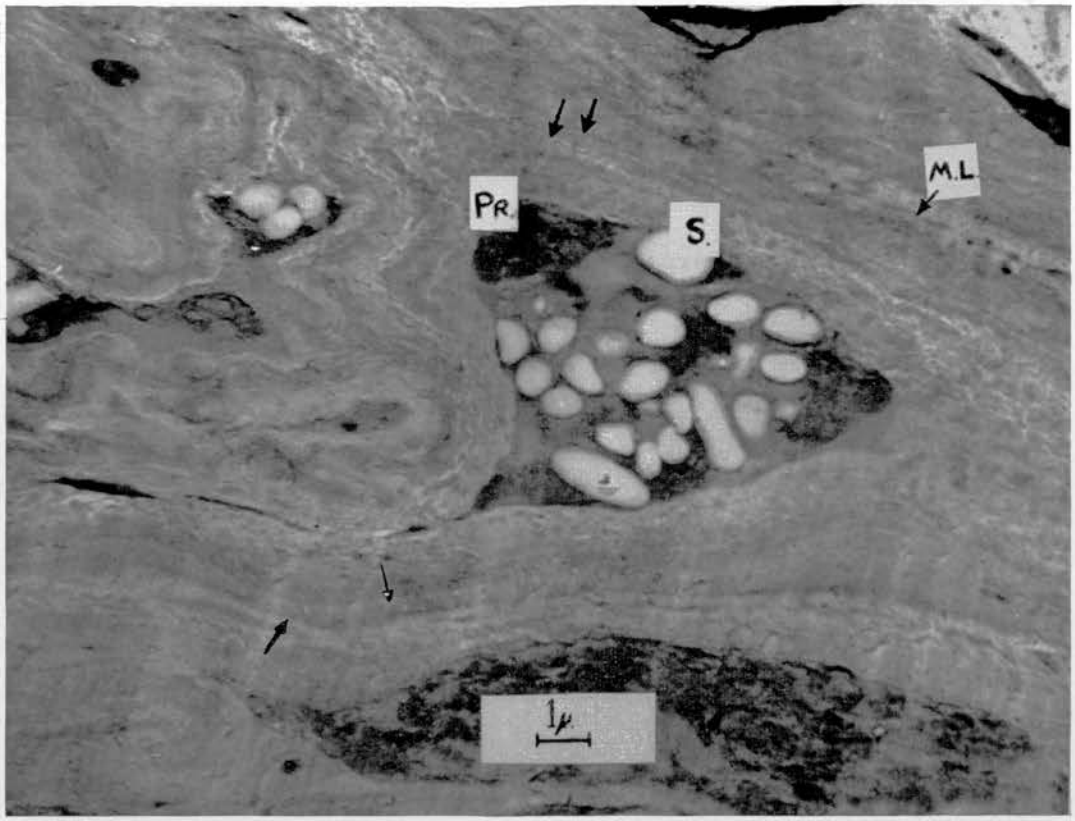
Fig. 3.—Barley aleurone.



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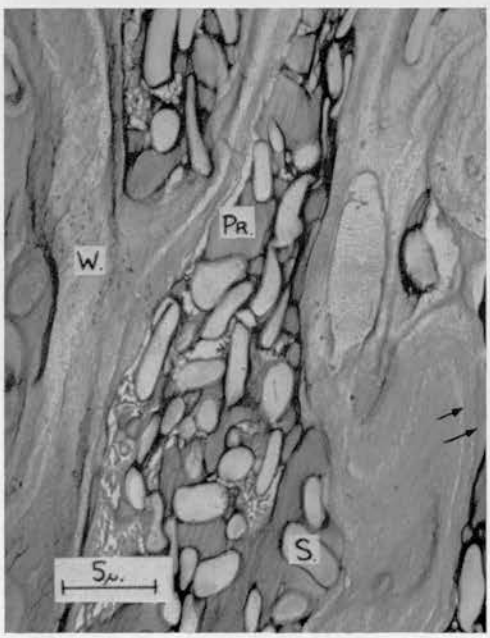

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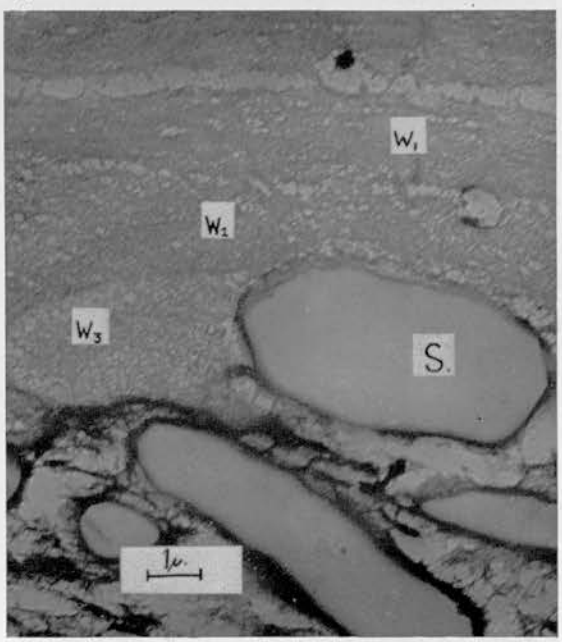

(*c*)

Fig. 4.—Starchy endosperm of *Bromus*.

For preparations from developing wheat caryopses, Buttrose[2] has additionally used fixation with osmic acid and araldite embedding, and the fine structure of aleurone from ripening wheat appears to be essentially similar to that now reported for barley and for *Bromus*. In wheat there are also unidentified bodies which vary in electron density at different stages of ripening and which become electron dense after steeping for 24 hr.: this variation is attributed by Buttrose to differences in degree of hydration. We do not feel that this is the whole explanation, at least for barley, since the U.B. from fully hydrated aleurone, maintained separately from the embryo, remain electron translucent after 24 hr.

However, as was indicated in the Introduction, it is not proposed to speculate extensively on the nature and function of any of the less familiar organelles detected in the aleurone. It is hoped that the significance of some at least of these inclusions will become clear when more detailed examination has been made of tissue from germinating or gibberellin-treated material. It is, nevertheless, reassuring to find that *Bromus* and barley yield essentially similar electron micrographs and that both contain aleurone cells which resemble those described by Buttrose for wheat.

Acknowledgements.—We should like to express our thanks to Mr. John Sheldon and to Mr. Simon Fairnie for technical assistance. One of us (J. H. D.) is particularly indebted to Plant Protection, Ltd., for a grant in aid of this work.

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XVII

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GIBBERELLIC ACID IN THE GERMINATION OF BARLEY

by

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INTRODUCTION

During the five years which have elapsed since the first comprehensive European study of gibberellic acid in malting[1], numerous descriptions have appeared of the characteristics of malts produced in response to amounts of gibberellic acid ranging from 0.001 to 3.0 mg/kg of barley. A survey of this work shows that there is a striking similarity in the effects reported: thus, a decrease in the time required for adequate modification is regularly observed and, along with this increase in the tempo of modification, there is frequently an enhancement of the activity of certain enzymes, notably the hydrolytic enzymes such as α-amylase, cytases and proteinase. The increase in proteolytic activity can be largely suppressed by adding bromate[2] or by adjusting growing conditions during malting[3], and a malt can be produced which analytically resembles malt made without added gibberellic acid, but which is finished approximately two days sooner than the untreated malt.

Economically these are important findings but, despite the unusual unanimity of opinion concerning the gross effects to be expected from controlled treatment of germinating grain, there is as yet no real understanding of the fundamental mode of action of gibberellic acid. Nor is there any satisfactory explanation of many other rather spectacular effects of gibberellins on plant metabolism — ability to substitute for red light in overcoming the dormancy of lettuce seed[4], transformation of peas from a dwarf to a tall habit of growth[5], induction of flower formation in certain long-day plants maintained in short-day conditions[6], and restoration of the juvenile leaf form in ivy[7] — all these and other responses to gibberellin treatment await satisfactory biochemical explanations.

The failure to discover an all-embracing first cause of the assorted phenomena associated with gibberellin treatment cannot be ascribed to any lack of experimental investigation, for even by 1958 STODOLA[8] had amassed over 1,000 references to publications on gibberellin, and experimental work on this group of hormones continues unabated. At first sight the physiological explanation underlying the appearance of gigantism in dwarf peas or rejuvenescence in ivy might seem to be rather remote from problems concerning enzyme development in malting; on the other hand, the primary activity of a growth factor may be similar in very different situations, though the manifestations of the result of that first stimulus can be expected to express themselves in different fashions according to the genetic constitution of the plant involved and the internal metabolic balance at the time of application.

Cumulative evidence from many sources has shown that the gibberellins constitute a group of natural plant hormones which are present in many unrelated flowering plants, and which are apparently particularly prominent in immature seeds[9]. Barley itself is a source of gibberellin[10,11]; immature grain does not seem to have been explored but, from analogy with that rich source of growth factors, the liquid endosperm of the coconut, it might be expected that the 'milky' stage of ripening grains would be worth exploring for gibberellins. Although the precise chemical nature of the endogenous gibberellin of barley is not yet known, the fact that one of the gibberellins is almost certainly present in the grain transforms the question of gibberellin treatment of malting barley from an applied problem concerned primarily with the effects of an additive into a fundamental study of major physiological importance. It is unlikely that added gibberellin behaves differently from the endogenous material of the grain, and the availability of pure gibberellic acid has thus simply made available a convenient source of a plant hormone. Gibberellins have now joined the exclusive group of chemically-characterised natural growth substances, whose principal member to date is indole acetic acid — a compound which rivals gibberellic acid in its versatility as a causal factor in morphogenesis and in the obscurity which surrounds its mode of action.

Identification of gibberellins

The characterisation of naturally-occurring growth substances is never an easy task. Apart from the difficulties imposed by the fact that plant hormones may occur in concentrations less than one part per million of dry weight of the tissue, there is the ever-present hazard of formation of artefacts, with hormonal attributes, during the extraction process. The predominantly polar transport of indole acetic acid allows this auxin to be collected by simple diffusion into agar blocks, but unfortunately gibberellins do not appear to be translocated within the plant body in one direction only: simple diffusional collection is therefore not possible and extraction by solvent becomes inevitable. Ethyl acetate treatment at pH 2.5 affords a satisfactory method of extraction for gibberellic acid[9], and considerable purification of the extracted material can be achieved by paper partion chromatography in a suitable solvent mixture. Thin-layer chromatography[12] has also provided a powerful tool for the separation of microgram quantities of gibberellins; with this technique, after separation and treatment with concentrated acid, any gibberellins present show a characteristic fluorescence in ultraviolet light. Chromatographic identity of movement and similarity of fluorescence are not rigid proofs of chemical identity but the regular appearance in a plant extract of material which behaves identically with gibberellic acid is at least presumptive evidence for the occurrence of gibberellic acid in that extract.

Another type of test which can be used to detect and, in favourable circumstances, to assay gibberellins in general and gibberellic acid in particular is provided by utilising a characteristic growth response. Several such biological tests have been developed[13,14], but again, a positive response to a biological test is best regarded as presumptive evidence for the presence of the substance being sought; it must be recognised that plant tissues contain a range of growth substances which have significant biological effects but which have not yet been isolated or identified.

Response of barley to gibberellic acid

Added gibberellic acid exerts its main influence on barley not through the the embryo but directly on the endosperm. The general locus of gibberellin stimulation was first established by YOMO[15] and, independently, by PALEG[16]. Later, MACLEOD AND MILLER[17] were able to show that the starchy cells of the endosperm do not respond to gibberellic acid if they are freed from all traces of aleurone by dissection, and that no response is shown if the aleurone has deteriorated in respiratory powers to such an extent that it fails to reduce tetrazolium salts. BRIGGS[18] has also concluded that a living aleurone is required for response to gibberellic acid: his approach to the problem involved killing the isolated endosperm by treatment with ether and noting the loss of response to gibberellin after this treatment. Among them, these four groups of workers have used as criteria of gibberellin-induced activity almost all of the major effects observed in barley malted with added gibberellic acid – development of sugars in the endosperm, production of α-amylase and of the cytolytic enzymes, endo-β-glucanase and pentosanase, and increase in soluble nitrogenous compounds in the endosperm. All have suggested that, in barley germinating without added gibberellin, the embryo supplies to the endosperm some material with gibberellin-like properties which is essential for the chain of degradative reactions required to provide soluble nutriment for the growing seedling: with the reports of gibberellin in barley, the deduction that the gibberellin-like substance might in actual fact be a genuine gibberellin seemed not unwarranted. Proof of the participation of the endogenous gibberellin in germination is as yet lacking, though YOMO's observation[19] (which has been confirmed by BRIGGS[18]) that the culture of separated embryos and endosperms in one vessel causes the formation of more amylase than does the culture of the two tissues separately clearly points to the interaction of embryo and endosperm in enzyme production.

Mode of action of gibberellic acid

Two broad possibilities exist regarding the mechanism by which gibberellic acid catalyses the secretion of hydrolytic enzymes to the central endosperm: either the hormone induces a release of enzymes from a bound condition or it stimulates the synthesis of the relevant enzymes from an amino acid pool. The first alternative was suggested by MACLEOD AND MILLER[17], with the caveat that it was merely hypothetical, while the second was strongly championed by BRIGGS[18] largely on the basis of results derived from treatments with metabolic inhibitors in conjunction with gibberellic acid.

The experimental work reported below was designed with two ends in view: first, to attempt to relate the behaviour of the enzyme-stimulating component of the embryo to the chemically-defined gibberellins, and secondly to explore more fully the actual mechanism of gibberellin-induced enzyme production.

Most of the techniques used have been described elsewhere[17]. Broadly the experimental approach involved the use of endosperm slices cut aseptically from husk-free grain; assessment of response to gibberellic acid was made by measuring the activity of endo-β-glucanase produced in the endosperm in response to the hormone. This cytolytic enzyme was selected for study partly because it is believed to be of real

significance in initiating cell-wall modification in the endosperm, and partly because other groups of workers[15,16,18] are already engaged in extensive studies of the response of endosperm expressed in amylase production.

EXPERIMENTAL — PART I

Endogenous gibberellin of barley

If added gibberellic acid is acting mainly as a supplement to the normal endogenous gibberellin believed to be present in the grain, then it is reasonable to expect that the magnitude of response to added gibberellin would be of the same order as that eventually developed in the endosperm maintained in contact with its embryo, but not subjected to any supplement. Many reports, however, suggest that in malting conditions formation of enzymes in response to added gibberellin is not only more rapid but also more extensive than that observed in untreated grain. On the other hand, it must be remembered that malting conditions may not always allow the grain to develop its full enzyme potential, as conditions towards the end of the malting period are gradually deteriorating from a biological point of view, with accumulation of CO_2 and diminution of available water leading to withering of the seedling and, presumably, sub-optimal conditions for metabolism in the aleurone. That the total development of endo-β-glucanase may be similar in extent in gibberellic acid treated corns and in excised endosperms maintained in sterile conditions in presence of gibberellic acid is seen from the results shown in Table 1, where comparison is made between

TABLE 1

TOTAL ENDO-β-GLUCANASE ACTIVITY OF ENDOSPERM

Barley variety	*Endosperm from intact corns (Grown 72 h)*	*Endosperm excised and supplied with 5 p.p.m. gibberellic acid (72 h)*
Wisa	306*	287
Wisa	344	279
Ymer	359	346

* Arbitrary units derived from effect on viscosity of a standard solution of barley β-glucan.

enzyme development in the endosperm of Wisa and Ymer barley after 72 h growth with adequate aeration and moisture, and enzyme development in isolated endosperms supplied with 1 p.p.m. of gibberellic acid for 72 h. Growth was at 25°C, a temperature which had previously been found to give maximum seedling growth, and which was known to be near the optimal for gibberellin induced sugar release; in these circumstances, very similar yields of the cytolytic enzyme were obtained from the growing corn and from the gibberellin-treated endosperm. Apparently then, in appropriate circumstances added gibberellin simulates, quantitatively as well as qualitatively, at least one of the changes characteristic of unaided germination.

It will be noted that the term 'total endo-β-glucanase' is used to designate the enzyme estimated in these experiments. The values for enzyme activity were obtained by grinding the endosperms in presence of the ambient fluid, together with sodium chloride added to a concentration of 0.6%; they thus include glucanase secreted to

the surrounding liquid, water-soluble glucanase retained in the endosperm, and salt-soluble glucanase.

Comparison of the glucanase content of excised endosperms which had been maintained in water at 25°C for approximately 3 days with that of excised endosperms subjected to the action of gibberellic acid for the same length of time showed that substantial amounts of β-glucanase could be extracted from the untreated endosperms by 0.6% NaCl, though no large amounts of enzyme were liberated into the surrounding fluid, nor was there any significant release of reducing sugar to the medium (Table 2). As has been shown earlier[17], the extensive secretion of sugars from the gibberellic acid treated tissue includes contributions from the activities of both the cytolytic systems, glucanase and pentosanase. From the results presented in Table 2 it would appear, therefore, that the endosperm in isolation develops a potential of endo-β-glucanase activity which is released on the advent of gibberellic acid, but that the cytolytic enzymes do not act on the hemicelluloses of the endosperm until germination supervenes or until exogenous gibberellic acid is admitted to the aleurone. The increasing extractability of endo-β-glucanase from untreated endosperm slices as incubation is prolonged from 24 h to 68 h does not appear to be due to influence from the embryo during the 4 h stand in 50% acid which was employed as a means of dehusking the grain, as slices which were cut from unsoaked grain peeled by hand and sterilised with hypochlorite gave essentially the same results (Table 2).

TABLE 2

SOLUBILITY OF ENDO-β-GLUCANASE

	Endosperm slices + water (25°C; 68 h)	*Endosperm slices + gibberellic acid (25°C; 68 h)*
Enzyme† secreted to surrounding fluid	20	290
Enzyme extracted by 0.6% NaCl	450	520
Total enzyme	470	810
Reducing sugar excreted*	1	60

* mg apparent glucose per 100 mg of endosperm.
† Arbitrary units.

Gibberellin-like material in barley endosperms

Although a latent potential of endo-β-glucanase may be present in the endosperm of ungerminated grain, expression of the activity of this enzyme involves the participation of a respiring aleurone[17]. That the aleurone is implicated in transmission of the enzyme-stimulating hormone as well as in response is easily shown by making incisions peripherally into the grain in such a way that the aleurone is severed completely while the dead cells of the central endosperm are left largely in contact with the scutellum. After such treatment the distal region of the endosperm, isolated by the incision, fails to show the changes characteristic of malting unless gibberellic acid is supplied. Apparently the endogenous enzyme-stimulating material of the embryo is unable to bridge the narrow gap, though gibberellic acid supplied in aqueous solution can be absorbed by the cut aleurone. Mechanisms involved in translocation in plants are still a cause of considerable controversy, but it is possible that some of

the energy-requirement for response to gibberellin may be related to passage through the living cell membrane. However, before entering the controversial field of hormone transport, it is desirable to examine more closely the actual material which may undergo translocation from embryo to aleurone.

In an exploratory survey of the nature of this material, 500 embryos excised from a sample of Ymer barley were triturated with phosphate-citrate buffer, pH 2.5, and then extracted with ethyl acetate. The separated extract was taken to dryness under reduced pressure and the residue was transferred to ethyl alcohol and again dried. An aqueous solution of this residue — which was free from amylase activity — induced secretion of reducing sugars from slices of barley endosperms as shown in Fig. 1. It should be carefully noted that the curve showing response to authentic gibberellic acid is valid only for slices cut from the sample of Wisa barley used in the determinations. Other samples of Wisa or other variety, or the same sample tested 3 months later, gave different curves. It was hoped initially that this type of test might provide a suitable bioassay for gibberellic acid: however, although the test is sensitive, the range of gibberellic acid concentrations stretching from no response to maximal response is usually small, and the need to derive separate standard curves on each occasion on which the assay is carried out makes it too laborious for routine use. The lettuce hypocotyl extension test, devised by FRANKLAND AND WARING[14] and believed to be specific for gibberellic acid, is less sensitive but very much more convenient to use.

None the less, as reference to Fig. 1 shows, material extracted from 500 barley embryos excised from the grain after 2h steeping induced liberation of sugar from endosperm slices equivalent to the amounts released in response to 0.2 μg of gibberellic acid. When seedlings were excised after 4 days growth the activity of this gibberellin-like material had apparently declined to about one tenth of that originally present. This diminished response may be due to translocation of hormone away from the embryo, but the possibility that the results recorded simply reflect a changed balance of stimulators and inhibitors present in the embryo extract should not be

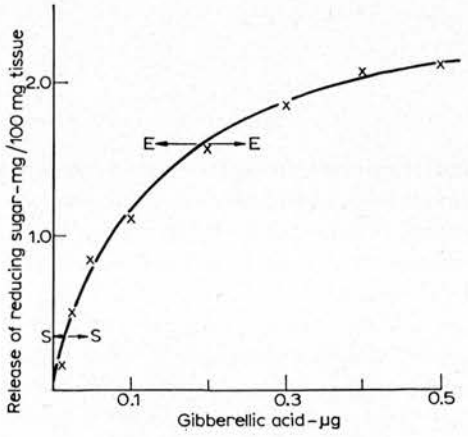

Fig. 1. Release of reducing sugar (as glucose) from endosperm slices in response to: gibberellic acid ×——× ; extract from 500 barley embryos E ←——→ E; extract from 500 4-day seedlings S ←——→ S. 23 h incubation at 25°C.

overlooked. However, when they are taken at face value, these results are in agreement with the hypothesis that, coincident with germination, gibberellic acid is secreted from embryo to aleurone; further study of possible gibberellin like materials in the endosperm of germinated grain is made difficult by the apparent presence therein of extractable growth-inhibiting material.

In addition to its effect on endosperm slices, the extracts of barley embryos gave a positive result in the lettuce hypocotyl extension test[14].

To supplement these bioassays of gibberellin-like material, use has also been made of thin-layer chromatography. With the methods used[12], and by considering Rf values in conjunction with fluorescence under ultraviolet light after treatment with sulphuric acid, discrimination could be made between gibberellic acid and almost all other known gibberellins. Again, suitable treatment of excised embryos has indicated that the principal gibberellin present in barley embryos is probably gibberellin A_3 – *i.e.* gibberellic acid itself. The technique used did not achieve distinction of gibberellic acid from gibberellenic acid and further study of separation is now in progress.

Again it must be emphasised that this technique does not provide an *identification* of the material from barley embryos as gibberellic acid: it demonstrates that barley embryos contain a component which in its movement in the appropriate solvent system and in its appearance in ultraviolet light after spraying with sulphuric acid behaves identically with gibberellic acid and differently from most other gibberellins.

In contrast to the extracts of embryos, where only one distinct mobile gibberellin-like component could be detected on thin-layer chromatograms, extracts from endosperms contained several components, none of which could be referred to the published data for known gibberellins[12]. These compounds are now under investigation, but whether they exhibit any growth-stimulating or growth-inhibiting propensities is as yet not known.

These three lines of evidence — the similarity of response as regards β-glucanase production whether the enzyme-inducing factor is provided by the embryo or supplied as authentic gibberellic acid; the extraction from the embryo of material with enzyme-inducing and growth-stimulating properties similar to those of gibberellic acid; and the chromatographic demonstration that a substance with certain properties identical with those of gibberellic acid can be prepared from barley embryos — strongly suggest that gibberellic acid is a naturally-occurring component of barley. The very simple demonstration with 'ringed' corns that severing the aleurone can halt modification and that application of gibberellic acid can re-establish the reactions leading to modification beyond the ring, further suggests that gibberellic acid is not only present in barley embryos but also essential for successful utilisation of the reserves of the endosperm.

EXPERIMENTAL — PART 2

Mode of action of gibberellic acid

One of the most popular and useful tools for unravelling the intricacies of a metabolic pathway is provided by the use of a carefully-chosen enzyme inhibitor. Stringent precautions must be observed both in the use of metabolic poisons and in inter-

pretation of results obtained in their presence, or quite unwarranted conclusions may be drawn. Consider, for example, the events set in motion by the introduction of gibberellic acid to slices of barley endosperm and culminating in the excretion of reducing sugars to the surrounding medium. In broad outline the train of events may be visualised as follows: (a) gibberellic acid in solution —→ (b) penetration through cell membrane —→ (c) reactions leading to mobilisation of hydrolytic enzymes —→ (d) hydrolysis of starch and other polysaccharides —→ (e) accumulation of reducing sugars.

If the effect of adding gibberellic acid is assessed by estimating the amount of reducing sugar liberated and is found to be abolished by a given inhibitor then, ideally, a determination should be made of the effect of that inhibitor on the uptake of gibberellin from solution and, routinely, an examination must be made of the effect of the inhibitor directly on the carbohydrases implicated in the eventual release of the sugars. No valid deductions can be made about the mechanisms involved in (c) if the inhibitor employed effectively poisons the hydrolytic enzymes concerned. Thus, the recorded[18] and undoubtedly correct observation that 2×10^{-3} *M* $HgCl_2$ and 2×10^{-3} *M* $CuSO_4$ abolish the response of endosperm fragments to gibberellic acid is not relevant to the problem since these concentrations of the salts also effectively inhibit β-amylase and partially inhibit α-amylase[20].

At the other end of the sequence, the possibility that a metabolic inhibitor may be impeding access of gibberellin to the site where it exerts its effects should not be forgotten, though this possibility is at present difficult to explore experimentally. However, the uptake of ions and of certain organic molecules is almost abolished by such metabolic inhibitors as chloramphenicol (2×10^{-3} *M*) and 2,4-dinitrophenol (10^{-4} *M*), and these established facts[21,22] should be kept in mind when an explanation is sought of the changing response to gibberellic acid in presence of certain inhibitors.

Since it has previously been shown that decline in ability to reduce tetrazolium salts and deprivation of oxygen are both associated with failure to respond to gibberellic acid[17], the effect of inhibitors which interfere with the oxidation of tricarboxylic acid (TCA) cycle intermediates and with terminal oxidases was examined. Also, since the operation of the TCA cycle is normally linked with the generation of adenosine triphosphate (ATP) from adenosine diphosphate, use was made of inhibitors which can uncouple respiration from ATP production, so depriving the tissue of the bond energy which is normally required for synthesis of proteins and other macromolecules. Finally, a survey was made of the effects of certain agents which are believed directly to inhibit protein synthesis.

For each assay, 10 2-mm slices cut from dehusked barley were incubated in 4 ml of water, or in 4 ml of water + gibberellic acid, or in 4 ml of water + gibberellic acid + inhibitor. Solutions of inhibitors were adjusted to pH 7 with NaOH or HCl, but no buffer was incorporated in the medium since, even at the dilutions employed, buffer mixtures may introduce further complications to an already complex situation. Endo-β-glucanase was extracted from the tissue and measured viscometrically, and when any degree inhibition was observed, the effect of the inhibitor on endo-β-glucanase itself was determined; unless stated otherwise, the various inhibitors used had no effect on the enzyme directly when used at concentrations up to ten times those employed in the assays. All results are means of three or more separate experiments.

(1) *Inhibitors of cytochrome oxidase and of the TCA cycle*

Both potassium cyanide and sodium sulphide at 5×10^{-2} *M* completely abolished the enhanced glucanase activity which characteristically occurs in response to gibberellic acid: although, at these concentrations both compounds typically inhibit cytochrome oxidase, they also inhibit endo-β-glucanase directly, so that no conclusions can be drawn from this effect. Sodium azide (10^{-2} *M*) had no effect on the enzyme directly, but at 10^{-4} *M* it inhibited the gibberellic acid response. Azide certainly interacts with metalloporphyrins such as cytochrome oxidase, but in dilute solutions (as used here) it can also operate as an uncoupling agent.

Sodium malonate, which is a well-known competitive inhibitor of succinic dehydrogenase, prevented the gibberellin-induced mobilisation of enzyme; however, this inhibition was not reversed by addition of succinate, as would be the case if the only effect of malonate was on the TCA cycle. The existence in barley seedlings of this unusual type of malonate-sensitive system has been noted previously[23] but the affected enzyme has apparently not yet been determined. These attempts to interfere with the usual path of electron transport have thus not yielded any firm conclusions and other potential reactants with intermediates in electron transfer are being sought. Fluoroacetate is a convenient inhibitor of the TCA cycle, and, as BRIGGS[18] has shown, it prevents the action of gibberellic acid on fragments of endosperm; however, interpretation of this effect presents some difficulty, for acetate buffer alone abolishes gibberellin-mediated sugar release[16] when it is supplied at 10^{-2} *M*. Endo-β-glucanase production is even more sensitive to the presence of acetate as its production is totally annulled by acetate buffer at 2×10^{-3} *M*: no study has therefore been made of possible effects of fluoroacetate.

(2) *Uncoupling agents*

The recognition that 2,4-dinitrophenol (DNP) at low concentrations inhibits plant growth by inhibiting oxidative phosphorylation has led to the frequent use of this and other 'uncoupling' reagents in metabolic studies. However, the dissociation of respiration from its normal accompaniment, the generation of energy-rich bonds, is not the only possible action of DNP. As SIMON[24] has clearly demonstrated, DNP at 10^{-5} *M* inhibits oxidative phosphorylation while simultaneously increasing O_2 uptake: at 10^{-4} *M* DMP inhibits aerobic respiration; and at 10^{-3} *M* it inhibits fermentation as well. To show that DNP is affecting *only* oxidative phosphorylation, it is essential to determine the concentration at which O_2 uptake is stimulated maximally: simple Warburg manometry shows that barley aleurone reacts to DNP in the same manner as do yeast and other plants[24]. To interpret the effect of DNP at 10^{-3} *M* as due to its uncoupling action[18] is thus not legitimate since, at this high concentration, many other facets of metabolism are also disturbed.

Results obtained with DNP at a concentration of 10^{-5} *M* and with sodium salicylate (5×10^{-4} *M*) are given in Table 3, and, as O_2 uptake was enhanced at this level of application, it may be assumed that there was no interference with aerobic respiration. Both DNP and sodium salicylate almost completely suppressed the expression of the endo-β-glucanase activity which is developed in water; presumably, then, the low level of activity which is observed in the absence of added gibberellin is not due to any pre-existing active enzyme present in the cut slices, but

TABLE 3

EFFECT OF INHIBITORS OF OXIDATIVE PHOSPHORYLATION ON GLUCANASE DEVELOPMENT

(18 h incubation at 25°C)

Incubation medium	*Endo-β-glucanase*†
Water	60
G.A.* (1.5×10^{-5} *M* to 1.4×10^{-3} *M*)	330
DNP** (10^{-5} *M*)	16
DNP (10^{-5} *M*) + G.A. (1.5×10^{-5} *M*)	163
DNP (10^{-5} *M*) + G.A. (2.3×10^{-4} *M*)	82
DNP (10^{-5} *M*) + G.A. (1.4×10^{-3} *M*)	58
Sodium salicylate (5×10^{-4} *M*)	12
Sodium salicylate (5×10^{-4} *M*) + G.A. (1.5×10^{-5} *M*)	171
Sodium salicylate (5×10^{-4} *M*) + G.A. (7.5×10^{-4} *M*)	46

* Gibberellic acid; **2,4-dinitrophenol; † Arbitrary units.

rather to a minimal activation which normally occurs during the incubation in water. Both the uncoupling agents can thus inhibit formation of endo-β-glucanase — and with both the inhibitory effect is partially overcome by adding 1.5×10^{-5} *M* gibberellic acid. At higher concentrations of gibberellic acid, the inhibitory effect of DNP and of salicylate is partially restored. Consideration was given to the possibility that this peculiar pattern of behaviour might be due to inadequate supplies of oxygen being available to the rapidly-respiring aleurone, with a possible deprivation of the mechanism involved in enzyme production, but as incubation in an atmosphere of O_2 did not affect the pattern of results, this possibility has been dismissed.

(3) *Inhibitors of protein synthesis*

With the rapidly increasing understanding of the probable mechanism underlying protein synthesis, biochemical methods are becoming available which can more or less specifically inhibit the synthesis of naturally-occurring proteins. Thus, certain amino acid analogues can be introduced to the cell and can compete with native amino acids in the formation of cell protein. If this protein normally functions as an

TABLE 4

EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON ENDO-β-GLUCANASE DEVELOPMENT

(18 h incubation at 25°C)

Incubation medium	*Endo-β-glucanase**
Water	60
Gibberellic acid (1.5×10^{-5} *M* to 1.4×10^{-3} *M*)	330
5-methyl tryptophan (5×10^{-3} *M*)	30
5-methyl tryptophan (5×10^{-3} *M*) + G.A. (1.5×10^{-5} *M*)	47
5-methyl tryptophan (5×10^{-3} *M*) + G.A. (1.5×10^{-4} *M*)	18
Chloramphenicol (3×10^{-3} *M*)	10
Chloramphenicol (3×10^{-3} *M*) + G.A. (1.5×10^{-5} *M*)	88
Chloramphenicol (3×10^{-3} *M*) + G.A. (7.5×10^{-4} *M*)	113

* Arbitrary units.

enzyme then, with the unnatural amino acid as a constituent part, the enzymic propensities may be impaired or totally abolished.

A second type of protein inhibitor, exemplified by the antibiotic, chloromycetin, appears to act by interfering in some manner with the formation of the macromolecule from its constituent amino acids: how this is accomplished is not known. Results obtained with one amino acid analogue, 5-methyl tryptophan, and with chloramphenicol are shown in Table 4. Both reagents virtually abolished the production of endo-β-glucanase in water, and added gibberellic acid had no apparent ability to restore — or, as is more usual in unpoisoned systems, — enhance the production of the enzyme in presence of 5-methyl tryptophan. With chloramphenicol, on the other hand, increasing the concentration of gibberellic acid to a large extent overcame the chloramphenicol-induced inhibition.

DISCUSSION

The results reported in the first part of this work require little further comment as they merely add weight to the general opinion that one of the gibberellins — possibly gibberellic acid itself — is intimately concerned in the production of hydrolytic enzymes in barley germinating without extraneous additions. The painstaking work of YOMO, in particular[15,19], has already gone far to establish this and, indeed, the principal outstanding requirement now is a chemical characterisation of gibberellin from barley.

Discussion of the effects reported with various metabolic inhibitors does seem to be desirable, first to suggest a possible explanation of some of the phenomena, and secondly to attempt to relate the present work to similar studies in progress elsewhere.

Of the various inhibitory effects observed two are of particular interest — the inhibition exercised by agents which impede protein synthesis, and the peculiar behaviour of certain uncoupling agents. The abolition of the response to gibberellic acid by 5-methyl tryptophan suggests that, for production of hydrolytic enzymes, active incorporation of amino acids into natural protein must be in progress — a point which has already been established by BRIGGS[18], who recorded a failure in the mechanism of sugar excretion from endosperm fragments maintained in ethionine or *p*-fluorophenylalanine + gibberellic acid. Ignoring for the moment the curious effect of chloramphenicol and considering only the action of the amino acid analogues, we may conclude that activity of a native protein is an essential concomitant of gibberellin-induced enzyme production. Caution is needed here, however, for it does not inevitably follow from this that the proteins whose biological efficiency is impaired are necessarily the hydrolytic enzymes themselves: other reactions, enzymic in nature, may occur between the provision of gibberellic acid to the endosperm slices and the release of enzymes, and a lesion in any one necessary enzyme would affect the working of the whole system. Again, it is dangerous to consider results in isolation, especially when a second type of protein inhibition gives rather different results. Thus, chloramphenicol also annulled the expression of gibberellin-induced activity in presence of low concentrations of gibberellin — but, with increasing availability of gibberellic acid this inhibiting effect of chloramphenicol was partially overcome.

Chloramphenicol can interfere with uptake of solutes, presumably through a

protein-inactivating mechanism at the level of the cell membrane[21]. Now, if we make two complementary assumptions, a reasonable explanation of some of the rather puzzling features mentioned above can be advanced. The assumptions are as follows:
(1) Gibberellin must enter the aleurone cell to exercise its effects; low concentrations of gibberellic acid may require an energy-assisted mechanism of intake, while higher concentrations may be conducive to entry by purely physical diffusion.
(2) Gibberellic acid increases membrane permeability.

Given assumption 1, we may deduce that, in circumstances where chloramphenicol has impaired active uptake at the cell membrane, gibberellic acid in concentrations of 2×10^{-5} *M* fails to enter the cell while, at higher levels of application, gibberellic acid can pass the barrier to uptake by simple physical diffusion and set in motion the chain of events associated with its presence.

Consider now the effects of DNP. With this inhibitor, increasing the concentration of gibberellic acid above the optimal level also increased the degree of inhibition. Working on assumption 2 — that gibberellic acid increases membrane permeability — we reach the conclusion that excess of gibberellic acid allows better penetration of DNP so that the inhibitor exercises its effects more completely within the mitochondrion. This suggestion is open to practical experimentation: if DNP is penetrating more effectively, then concentrations which normally inhibit only oxidative phosphorylation might be expected to exercise a more drastic effect and interfere also with oxygen uptake. Results obtained by Warburg manometry show that this is indeed so (Fig. 2): DNP alone or in presence of 1.5×10^{-5} *M* gibberellic acid is associated with increased O_2 uptake whereas the same concentration of DNP + 7.5×10^{-4} *M* gibberellic acid gives a marked decrease in O_2 uptake compared with the level observed in the untreated material.

This suggestion — that gibberellic acid may be associated with an increase in membrane permeability — is in harmony with the observed fact that movement of enzymes from the region of the aleurone either through the body of the endosperm or into solution in the surrounding fluid is greatly enhanced by the presence of gibberellic acid. The results given in Table 2 also point to the existence in untreated grain

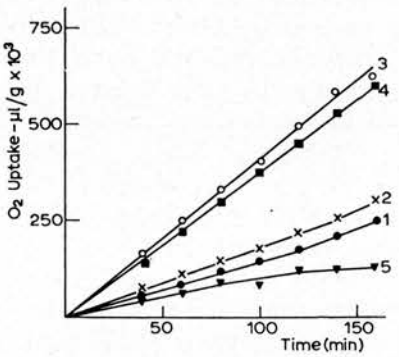

Fig. 2. Effect of gibberellic acid on inhibitory action of 2,4-dinitrophenol. Incubation media: 1, water; 2, 7.5×10^{-4} *M* G.A.; 3, 10^{-5} *M* DNP; 4, 1.5×10^{-5} *M* G.A. + 10^{-5} *M* DNP; 5, 7.5×10^{-4} *M* G.A. + 10^{-5} *M* DNP.
18 h incubation at 25°C in the given media, prior to measurement of O_2 uptake, by conventional Warburg technique.

of a considerable potential of endo-β-glucanase activity, present in steeped endosperm but unable to move into action in the absence of gibberellins.

The part played by protein synthesis in this reaction system remains obscure. Indeed, the extremely interesting results obtained recently by SRIVASTAVA AND MEREDITH[25] suggest that protein synthesis may be of little significance when relatively high concentrations of gibberellic acid are employed. Thus, malting barley grown in presence of 50 p.p.m. (ca. 1.5×10^{-4} *M*) gibberellic acid and ca. 3×10^{-2} *M* chloramphenicol showed a rate of α-amylase production which greatly surpassed that found in grain malted without additives and which was, indeed, virtually the same as that of grain malted without the inhibitor of protein synthesis. It seems, then, that we must accept with reserve the statement that gibberellic acid functions by initiating a *de novo* synthesis of hydrolytic enzymes — unless we can accept the fact that enzyme synthesis differs from other syntheses of proteins and is unaffected by massive amounts of chloramphenicol.

Many aspects of the reactions between gibberellic acid and germinating barley still await satisfactory explanations. The curious sensitivity to certain buffers[16], the inability of the aleurone to respond to gibberellin at temperatures above 37°C and the restoration of the typical response when the temperature is lowered[17] may be cited as features of interest in barley metabolism which require further examination. Biochemically speaking, we are still at the stage of speculating and erecting hypotheses: accumulation of experimental results will, in time, support or destroy some of these speculations and hypotheses. Allusion was made earlier to the varied effects of gibberellic acid on plant morphogenesis and to the complications inherent in hormone action: an excellent test of the validity of any explanation of the function of gibberellic acid in cereal germination might lie in examining the applicability of the explanation to all the other rather fascinating expressions of gibberellins in action. Whether we favour lysosome disruption[17], or induced synthesis of proteins[18], or alteration of membrane characteristics as major causes of the well-known effects on barley, we are still left with a very large unanswered question: how, at the molecular level, does a relatively simple molecule like that of gibberellic acid interact with the lysosome, or the protein-building organelle, or the membrane? Or are all these speculations wide of the mark?

ACKNOWLEDGEMENT

We should like to express our thanks to Professor I. A. PREECE for his helpful discussion of the results presented here, and for useful guidance on the preparation and manipulation of β-glucan and endo-β-glucanase.

SUMMARY

Although the results of many investigations into the overall effects of gibberellic acid on malting have shown that judicious use of gibberellin can accelerate modification and allow production of malts with higher extract potentials, little is known about the fundamental biochemical principles underlying these phenomena. In this paper results are presented of an investigation into the relationship between gibberellin-induced secretion of cell wall-degrading enzymes (especially endo-β-glucanase) and oxidative metabolism in the aleurone.

This aleurone layer is shown to be intimately concerned with modification in untreated barley: thus, if the aleurone of unsteeped grain is severed immediately above the embryo and the barley is then maintained in conditions suitable for germination, no cell wall modification takes place in the central endosperm above the incision although the scutellum is still in intimate

contact with the starch-filled cells. An "embryo factor" which acts only through the living aleurone is therefore essential for endosperm modification. This "embryo factor", which is as yet unidentified, can be replaced by added gibberellic acid: moreover, the amount of endo-β-glucanase developed in detached endosperms supplied with gibberellic acid is of the same order of magnitude as the amount formed in the endosperm of intact grains. This observation suggests that the "embryo factor" and gibberellic acid are, if not identical, at least very similar indeed in their metabolic effects.

Using the development of endo-β-glucanase activity in slices of endosperm as a criterion of response to gibberellic acid, a survey has been made of the effects of a number of inhibitors on the reaction:

$$\text{gibberellic acid} + \text{aleurone} \rightarrow \text{hydrolytic enzymes.}$$

It is shown that certain anti-metabolites, which are without effect on extracted endo-β-glucanase, can inhibit this chain of reactions partially or completely. These results, which must be interpreted with considerable caution, are discussed in relation to the general metabolism of malting.

LE RÔLE DE L'ACIDE GIBBERELLIQUE DANS LA GERMINATION DE L'ORGE

RÉSUMÉ

Bien que les résultats des nombreuses recherches sur les effets globaux de l'acide gibberellique au maltage aient montré qu'un emploi judicieux de la gibberelline peut accélérer la désagrégation et permettre la fabrication de malts possédant des extraits potentiels plus élevés, on connaît encore peu de chose sur les principes biochimiques fondamentaux qui régissent le phénomène. Nous allons présenter dans cet exposé les résultats de notre étude concernant la sécrétion provoquée par l'acide gibberellique des enzymes désagrégeant les parois cellulaires, notamment la β-endo-glucanase et ses relations avec le métabolisme oxydatif dans la couche à aleurone.

On montre que cette couche à aleurone est intimement associée à la désagrégation pour une orge non traitée: c'est ainsi que si on coupe immédiatement au-dessus de l'embryon, la couche aleurone d'un grain non trempé et qu'on maintienne ensuite l'orge dans des conditions convenables à la germination, il n'apparaît aucune désagrégation des parois cellulaires dans l'endosperme central au-dessus de l'incision, bien que le scutellum soit toujours en contact étroit avec les cellules garnies d'amidon. Nous en concluons qu'un "facteur embryon" n'agissant que par l'intermédiaire de la couche à aleurone vivante, est indispensable à la désagrégation de l'endosperme. Ce "facteur embryon", qui jusqu'à présent n'est toujours pas identifié, peut être remplacé par l'apport d'acide gibberellique; en outre, la quantité de β-endo-glucanase formée dans l'endosperme ainsi séparé, mais alimenté en acide gibberellique, est du même ordre de grandeur que la quantité formée dans l'endosperme des grains intacts. Cette observation nous conduit à penser que le "facteur embryon" et l'acide gibberellique sont, sinon identiques, du moins très voisins en ce qui concerne leurs effets sur le métabolisme.

En utilisant comme critère de la réaction de l'acide gibberellique:

$$\text{acide gibberellique} + \text{aleurone} \rightarrow \text{enzymes hydrolytiques}$$

le développement de l'activité de la β-endo-glucanase dans des tranches d'endosperme, nous avons fait une étude d'ensemble sur les effets d'un certain nombre d'inhibiteurs de cette réaction.

Nous avons démontré que certains anti-métabolites, qui sont sans effets sur la β-endo-glucanase extraite peuvent inhiber la chaîne de réactions partiellement ou même complètement. Ces résultats, qui doivent être interprétés avec la plus grande prudence, sont ici discutés pour autant qu'ils recoupent le métabolisme général du maltage.

DIE GIBBERELLINSÄURE BEI DER KEIMUNG DER GERSTE

ZUSAMMENFASSUNG

Obwohl die Ergebnisse zahlreicher Untersuchungen über die Gesamtwirkungen der Gibberellinsäure auf das Mälzen gezeigt haben, dass eine vernünftige Anwendung von Gibberellin die Lösung beschleunigen und die Herstellung eines Malzes mit höherem Extrakt ermöglichen kann, ist über

die wesentlichen biochemischen Prinzipien, die diesen Erscheinungen zugrundeliegen, wenig bekannt. In dieser Arbeit werden Ergebnisse einer Untersuchung wiedergegeben über die Beziehung zwischen der durch Gibberellin bewirkten Ausscheidung zellwandabbauender Enzyme (besonders der Endo-β-Glukanase) und dem oxydativen Stoffwechsel in der Aleuronschicht.

Es wurde gezeigt, dass diese Aleuronschicht mit der Lösung unbehandelter Gerste in engem Zusammenhang steht; wenn also das Aleuron ungeweichter Gerste unmittelbar über dem Keimling abgetrennt und die Gerste dann in Bedingungen gehalten wird, die eine Keimung ermöglichen, findet oberhalb des Einschnitts in der Mitte des Mehlkörpers keine Zellwandlösung statt, obwohl das Schildchen noch in engem Kontakt mit den mit Stärke gefüllten Zellen steht. Ein "Keimlingsfaktor", der nur durch das lebende Aleuron wirkt, ist deswegen für die Mehlkörperlösung essentiell. Dieser "Keimlingsfaktor", der bis jetzt noch nicht identifiziert wurde, kann durch zugefügte Gibberellinsäure ersetzt werden; darüberhinaus liegt die Menge der Endo-β-Glukanase, die in abgetrennten Endospermen mit Gibberellinsäurezusatz entwickelt wird, im selben Grössenbereich wie die im Mehlkörper unangetasteter Körner gebildete Menge. Diese Beobachtung führt zu der Annahme, dass der "Keimlingsfaktor" und Gibberellinsäure, wenn nicht identisch, so doch zumindest in ihren Wirkungen im Stoffwechsel tatsächlich sehr ähnlich sind.

Unter Verwendung der Entwicklung der Endo-β-Glukanase-Aktivität in Endospermschnitten als Kriterium für die Reaktion auf Gibberellinsäure wurde eine Prüfung der Wirkung einer Reihe von Hemmstoffen auf die Reaktion:

Gibberellinsäure + Aleuron → hydrolytische Enzyme

vorgenommen. Es wird gezeigt, dass gewisse Antimetaboliten, die ohne Wirkung auf extrahierte Endo-β-Glukanase sind, die Kette von Reaktionen teilweise oder vollständig unterbinden können. Diese Ergebnisse, die mit beträchtlicher Vorsicht zu interpretieren sind, werden im Zusammenhang mit dem allgemeinen Stoffwechsel des Mälzens diskutiert.

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DISCUSSION

Dr. R. SCRIBAN (France): Je désire souligner toute l'importance du travail de Miss MacLeod et apporter quelques éléments complémentaires sur le rôle joué par l'assise protéique (couche à aleurone) de l'orge:

(a) *d'ordre histologique*: la vacuolisation des cellules de l'assise protéique et leur évolution histochimique (variation de la métachromasie) est de plus en plus accentuée au fur et à mesure

qu'on approche du scutellum. Cela sourligne l'activité exercie par l'assise protéique au cours de la germination;

(b) *d'ordre histochimique*: l'assise protéique est particulièrement riche en amylase, en protéinase et en enzymes de la transamination qui interviennent dans la désagrégation du malt et la nutrition de l'embryon.

Dr. I. C. MacWilliam (Greet Britain): Experiments which we have carried out with isolated embryos and endosperms placed on either side of a dialysis membrane have allowed the "embryo factor" which Dr. MacLeod mentions to pass through the membrane from the embryo to stimulate the endosperm but do not allow enzymes to migrate. Estimations of the α-amylase formed on each side of the membrane at the end of a period similar to that of normal malting reveal that 80% of the total α-amylase produced has its origin in the aleuron layer and only 20% in the embryo. Of the latter half is transmitted to the endosperm during malting and half is retained in the embryo. These figures for α-amylase are similar to those for glucanase found by Dr. MacLeod in the whole barley and separated endosperm.

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DEVELOPMENT OF HYDROLYTIC ENZYMES IN GERMINATING GRAIN

BY

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Studies of the order and rates of production of certain hydrolytic enzymes in the endosperm of germinating barley and in endosperm slices treated with gibberellic acid have shown that endo-β-glucanase formation precedes the formation of α-amylase which, in turn, precedes the formation of protease. A rise in acid phosphatase appears to take place shortly after the induction of endo-β-glucanase. Although the order of induction of the enzymes is the same in slices and in grain germinating without additives, enzyme production begins some ten hours earlier in the slices. The response of isolated aleurone to gibberellic acid is qualitatively similar to that of the endosperm slices, but, though there is appreciable formation of enzymes, secretion is very slight. This suggests that the effect of gibberellic acid on hydrolase synthesis can be distinguished from any effect on secretion. Preliminary hydration of endosperm slices induces a much more rapid response to gibberellic acid.

Introduction

One of the most important events in malting is the development of an assortment of hydrolytic enzymes which exercise their effects during germination and, in some instances, later in the mash tun. It has long been known that several of the hydrolytic enzymes—notably α-amylase, endo-β-glucanase and proteinase—increase spectacularly in amount on the second or third day after steeping, in a traditional type of malting, and it has recently been shown that the tissue of the grain responsible for the immediate production of some of these hydrolytic enzymes is the aleurone layer of the endosperm. To induce the aleurone to produce hydrolytic enzymes and to secrete them into the adjoining starchy endosperm there must

be present either (*a*) an embryo supplied with water and oxygen and capable of growth or (*b*) an exogenous supply of gibberellic acid. It is possible that these are not valid alternatives and that the embryo may control the production of enzymes by secreting gibberellic acid—known to be present in immature barley[3]—to the aleurone. Exactly how gibberellic acid "switches on" the enzymes in the aleurone is still a mystery, though interesting hypotheses regarding its mode of action have recently been formulated.[9]

In view of the apparently close relationship between gibberellic acid, aleurone and hydrolytic enzymes, a comparative survey has been made of the course of development of four hydrolytic enzymes—α-amylase, endo-β-glucanase, proteinase and acid phosphatase—in the endosperm of germinating grain and in endosperm slices and isolated aleurone treated with gibberellic acid. The changes observed in enzyme potential in the variously treated tissues will later be compared with changes in fine structure observable in electron micrographs of the grain. For comparative studies with results of electron microscopy, determinations have also been made of two of these enzymes (α-amylase and endo-β-glucanase) in germinating caryopses and in endosperm slices of the grass, *Bromus inermis*.

Experimental Methods

The barley used throughout was a sample of Proctor from the 1963 harvest. The grain was dehusked by treatment with sulphuric acid and stored in a sterile container at 4° C. *Bromus inermis* was kindly supplied by Dr. W. O. S. Meredith of the Grain Research Laboratory, Winnipeg, and caryopses of *Bromus* were also dehusked before use.

Germinating grain.—Husk-free corns were placed in a 9-cm. petri dish containing filter paper saturated with distilled water and were left to grow at 25° C. At suitable times samples were taken: 2-mm. slices were then cut transversely from the endosperm 2 mm. behind the scutellum, and ground in 5 ml. of 0·1-M NaCl. The homogenate was kept for 2 hr. with occasional stirring at room temperature, the particulate material was then removed by centrifugation and the supernatant used for enzyme assay.

Slices treated with gibberellic acid.—2-mm. slices were cut from dry dehusked grain, weighed and incubated at 25° C. in 5-cm. petri dishes containing 4 ml. of 10^{-5} M gibberellic acid. Prior to grinding, 1 ml. of 0·5-M NaCl was added to the medium and thereafter extraction was carried out in the same manner as was used with slices cut from germinating grain.

Isolated aleurone.—Halved endosperm slices were steeped overnight in distilled water at 25° C. and then ground gently by hand in a glass homogenizer till the starchy cells were suspended above the aleurone fragments, which remained intact. The aleurone was separated from debris by sieving and washing and the final product, which was microscopically free from starch, consisted of apparently undamaged aleurone cells which retained the capacity to reduce tetrazolium salts and which consumed oxygen at a rate similar to that of intact slices containing equivalent amounts of aleurone. For this comparative work it would have been preferable to use aleurone isolated from dry grains but the walls fragmented when dry material was used and no clear separation of tissues could be made. The isolated aleurone was incubated in 10^{-5}-M gibberellic acid for various lengths of time at 25° C. and then enzyme activity was estimated in homogenates of the tissue and in the medium. The tissue was completely homogenized by grinding in a *Micro-Wet* grinder in the presence of ground glass. Solids were removed immediately by centrifugation and the extract was assayed without further delay.

Enzyme assay.—Endo-β-glucanase was determined viscometrically by the method of Preece & Hoggan,[7] using barley β-glucan as substrate; protease was also determined viscometrically by the method of Massart[8] with gelatin (pH 5) as substrate. Acid phosphatase and α-amylase were estimated colorimetrically, the former by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate and the latter by the iodine-dextrin colour method of Briggs.[1]

Results

Barley

The course of development of the four different hydrolytic enzymes in endosperm of germinating barley and in endosperm slices or isolated aleurone treated with gibberellic acid is shown in Fig. 1–4. All results have been quoted as units of activity for fresh weight of original endosperm, and each point

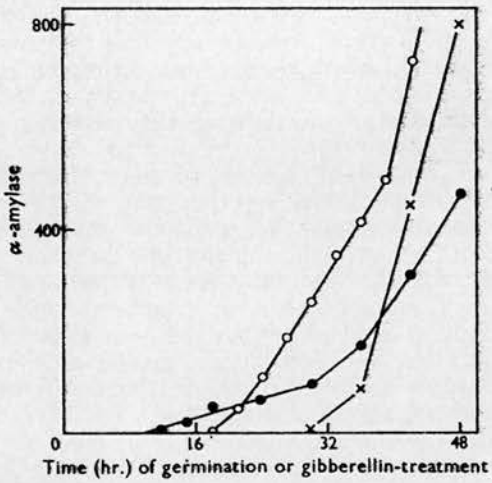

Fig. 1.—Development of α-amylase in barley. ○—gibberellin-treated endosperm slices; ●—gibberellin-treated isolated aleurone; ×—endosperm from germinating grain. Enzyme units: I.D.C. units per g. endosperm.

is the mean of at least three separate determinations. The 10 endosperm slices normally used per determination had a mean weight of 0·1830 g. with a standard deviation of ± 0·0129; this mean figure was taken as the basal value for slices cut from germinating corns, in which direct determinations of original weight could not be made.

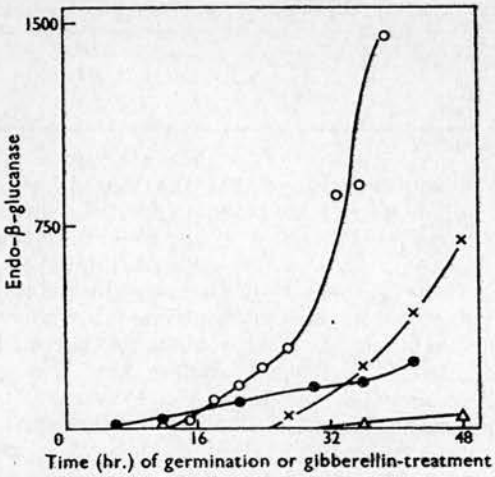

Fig. 2.—Development of endo-β-glucanase in barley. ○—gibberellin-treated endosperm; ●—gibberellin-treated isolated aleurone; △—enzyme secreted from gibberellin-treated isolated aleurone; ×—endosperm from germinating grain. Enzyme units: $\Delta^1/_{\eta\ sp.}$ per 100 g. per hr.

Compare first the general progress of development of hydrolytic enzymes in the endosperm of germinating grain with enzyme development in gibberellin-treated slices. The shapes of the curves for enzyme activity

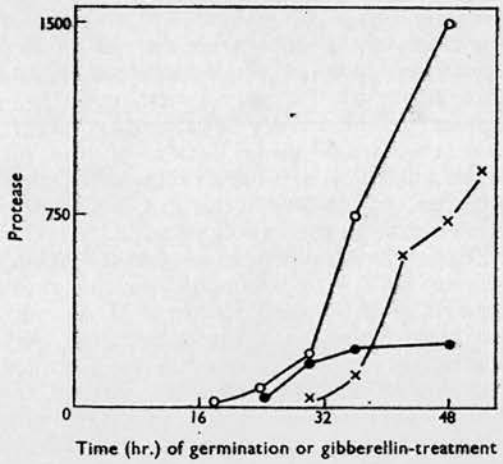

Fig. 3.—Development of protease in barley. ○—gibberellin-treated endosperm; ●—giberellin-treated isolated aleurone; ×—endosperm from germinating grain. Enzyme units: — △ η sp. × 10^{-3} per g. per hr.

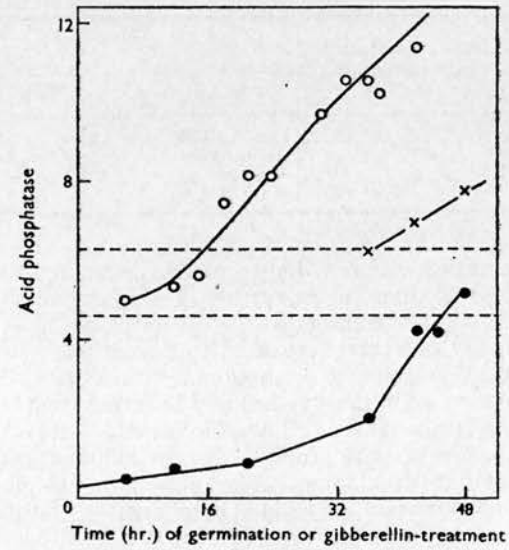

Fig. 4.—Development of acid phosphatase in barley. ○—Gibberellin-treated endosperm; ●—gibberellin-treated isolated aleurone; ×—endosperm from germinating grain. The dotted lines indicate the limits of variability of acid phosphatase in ungerminated grain. Enzyme units: mM *p*-nitrophenol per 100 g. per hr.

in endosperm are in general similar in the two situations, but, with each enzyme under consideration, the onset of rapid production is significantly later in the germinating grain than it is in the treated slices. With α-amylase, endo-β-glucanase and protease the apparent onset of enzyme production is approximately 10 hr. earlier in the treated slices than it is in the endosperm of the germinating intact grain; with acid phosphatase it is apparently 20 hr. earlier. Apart from the delay in initiation of enzyme formation, there are only minor differences in the pattern of development of each hydrolytic enzyme in the two types of material.

Enzyme development in response to gibberellic acid in isolated strips of aleurone shows some rather interesting features. Thus, only with endo-β-glucanase (Fig. 2) was there any measurable *secretion* of enzyme from purified aleurone to ambient medium, within the 48-hr. experimental period, though with slices containing both aleurone and starchy cells there is abundant secretion of endo-β-glucanase and α-amylase to the medium in only 18 hr. of incubation. This failure in secretion was not due to physical damage to the aleurone cells during grinding: when slices were treated with gibberellic acid for 24 hr. and then separated into aleurone and starchy cells, the aleurone continued to secrete α-amylase vigorously both into water and into 10^{-5} M gibberellic acid. Various additions were made to the incubation medium, including inorganic salts, tissue culture media, vitamin B components, amino acids, soluble starch and freeze-dried extracts of starchy endosperm, but none was able to promote rapid secretion of hydrolytic enzymes from the separated aleurone, in presence of gibberellic acid. Aleurone separated enzymically from starchy endosperm was also deficient in secretory powers: the best that could be achieved was a release of 48 I.D.C. units* per g. of tissue after 48 hr. This compares very poorly with results from corresponding whole slices, from which at least 2000 I.D.C. units of α-amylase are regularly secreted in this length of time.

It is also interesting to note that the magnitude of enzyme production within the isolated aleurone was generally much less than in treated slices. That the discrepancy between the two sets of figures is not simply due to loss of aleurone fragments during grinding and sieving can be seen from the fact that the percentages of the different enzymes recorded at different times in the two treatments did not vary consistently with the different enzymes (Table I).

If aleurone were simply being lost in processing, it would be expected that equivalent amounts of each enzyme would also be lost with the cells: it is clear from Table I that this is not the case. Moreover, in the early stages of incubation, the isolated aleurone apparently contained *more* α-amylase than did the slices. This phenomenon will be discussed later.

TABLE I

ENZYME IN ISOLATED ALEURONE AS % ENZYME IN COMPLETE SLICE

Time of incubation in gibberellic acid (hr.)	Enzyme			
	α-Amylase	Endo-β-glucanase	Protease	Acid phosphatase
20	165	68	—	50
30	38	34	80	38
40	46	15	33	50

It will be noticed that the four different enzymes do not all begin to increase simultaneously. The times of apparent initiation of three of the enzymes concerned are shown in Table II. Endo-β-glucanase production appears to get under way some 8 hr. before the beginning of the rise in α-amylase, which in turn is detectable before the rise in protease; these time-gaps are similar for gibberellin-treated slices and for endosperm from germinating corns, though they are 10 or more hr. later in the growing corns. The results recorded with acid phosphatase do not fit so tidily into this picture: the tindications are that the induction of

* I.D.C. = Iodine dextrin colour units.

TABLE II

TIME* OF INITIATION OF ENZYMES

Enzyme	Endosperm slices	Germinating grain
Endo-β-glucanase	12	24
α-Amylase ..	18	29
Protease	20	30

* Hr. of treatment with gibberellic acid or of germination.

phosphatase increase in treated slices begins at about 15 hr.—*i.e.*, just after the onset of endo-β-glucanase increase, and that, in germination, the phosphatase is the last of the four enzymes to show any marked rise. However, the rather high basal level of phosphatase in the untreated material (Fig. 4) may be obscuring early changes in activity.

Effect of hydration.—Although results obtained with strips of isolated aleurone have been included in Figs. 1–4 along with results from germinating grain and from endosperm slices, it will be realized that the metabolic status of the separated aleurone is here not really comparable with that of the other experimental material since the aleurone, attached to the rest of the endosperm, was steeped in water for 18 hr. before separation and treatment with gibberellic acid. Accordingly, analyses were made to determine if different periods of pre-incubation in water had different effects on subsequent enzyme development in the two principal regions of the slices—the aleurone and the starchy cells. Endosperm slices were steeped either for 18 hr. or for 24 hr. and then transferred to 10^{-5}-M gibberellic acid at 25° C. After an appropriate time interval, the slices were removed and separated into aleurone and starchy tissues and α-amylase activity was determined in each portion of the slice. Results of these determinations are shown in Fig. 5. It is clear that the slightly longer period of treatment with water has allowed the grain to develop a greatly enhanced potentiality for response to gibberellic acid. It seems probable, therefore, that the accelerated formation of enzyme in isolated aleurone compared with entire slices is a consequence of the unavoidable pre-treatment with water given to the separated aleurone.

The rate of production of enzyme in the two portions of the endosperm is of interest in that there is a very brief time lag—of the order of 3–6 hr.—between the first sign of α-amylase production in the aleurone and its appearance in the inner endosperm. The build-up of α-amylase in the starchy endosperm is remarkably rapid, especially in material which had been pre-soaked for 24 hr. where a more than fourfold increase occurred between 12 and 16 hr. after introduction of gibberellic acid.

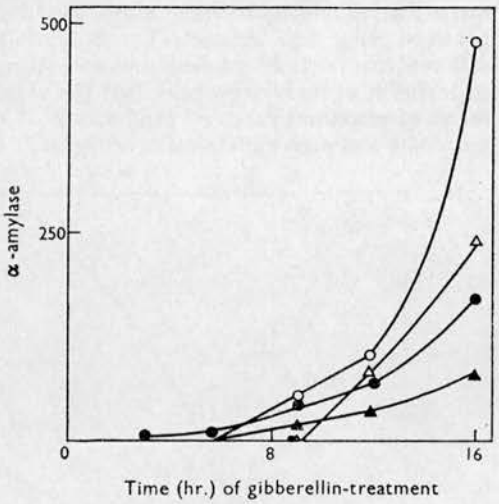

Fig. 5.—Effect of hydration on development of α-amylase in barley. ▲—enzyme in aleurone, 18 hr. pre-soaking. △—enzyme in starchy endosperm, 18 hr. pre-soaking. ●—enzyme in aleurone, 24 hr. pre-soaking. ○—enzyme in starchy endosperm, 24 hr. pre-soaking. Enzyme units: I.D.C. units per g. endosperm.

Bromus

Preliminary work showed that seeds of *Bromus* spp. responded to gibberellic acid in the same general manner as barley. Thus, hydrolytic enzymes were released rapidly into the endosperm of germinating grain but no rise in enzyme content occurred when isolated endosperms were incubated in water. When gibberellic acid was added to the incubation medium, however, hydrolytic enzymes rose significantly. As was the case with barley, no response to gibberellic acid was shown by fragments of starchy endosperm of *Bromus* and presence of active aleurone was a prerequisite for gibberellin-induced enzyme production in the endosperm.

The course of development of α-amylase and of endo-β-glucanase in germinating *Bromus* and in endosperm fragments treated

with gibberellic acid is shown in Figs. 6 and 7. Again the similarity between formation of enzymes in germinating grain and in gibberellin-treated slices will be noted and again the response of slices to added gibberellin occurred several hours before the response of the endosperm of intact seeds to a "germination" stimulus. In *Bromus*, as in barley, endo-β-glucanase production appears to be initiated before production of α-amylase. No quantitative comparisons can be made between the two grasses, because of the different proportions of aleurone and starchy endosperm in their caryopses, but the attainment of maximal rates of production of the enzymes was strikingly similar in both.

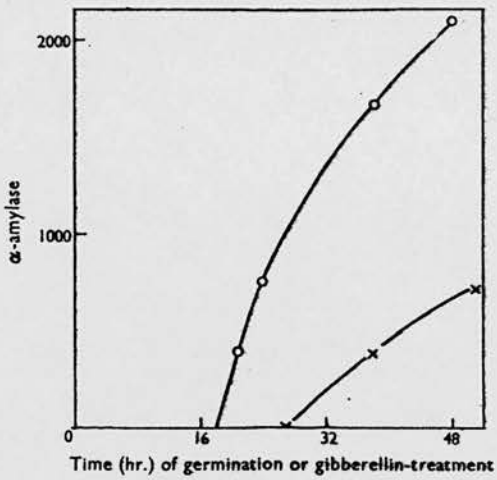

Fig. 6.—Development of α-amylase in *Bromus*. ○—gibberellin-treated endosperm. ×—endosperm from germinating grain. Enzyme units: I.D.C. units per 100 mg. endosperm.

Discussion

The ultimate aim of this work is to discover the *modus operandi* of gibberellic acid in inducing enzyme formation in germinating barley. The results reported here do not shed light on possible biochemical mechanisms involved; they do, however, provide a very clear illustration of the essentially similar behaviour of endosperm modifying under the influence of its own embryo and endosperm which is induced to modify by addition of gibberellic acid. Moreover, consideration of these results strongly suggests that attention should now be concentrated on exploring mechanisms initiated during the first few *hours* after application of gibberellic acid to

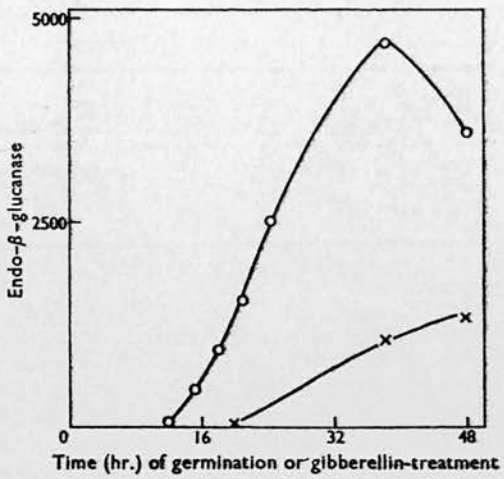

Fig. 7.—Development of endo-β-glucanase in *Bromus*. ○—gibberellin-treated endosperm; ×—endosperm from germinating grain. Enzyme units: $\Delta^1/_{\eta\ sp.}$ per 10 g. per hr.

slices: endo-β-glucanase is quite unequivocally increasing in activity 12 hr. after introduction of gibberellic acid to the aleurone of dry slices of endosperm, and it is reasonable to assume that there are important metabolic events which precede this first detectable sign of the presence of gibberellic acid. As well as examining the effects of gibberellic acid, there is a very clear need to study closely the effects of hydration on the aleurone, in view of the fact that pre-treatment with water (Fig. 5) advances the response to gibberellic acid by several hours.

It is difficult to make useful comparison of results reported here with the findings of other workers, because of extensive differences in treatment of the grains, but a number of general points appear to be worth making. Thus, Yomo & Iinuma[11] have also separated aleurone from starchy cells of barley by treatment of endosperm fragments for 3 days with cytolytic enzymes prepared from the fungus, *Trichoderma viride*; they then determined the activities of various hydrolases after exposure of the aleurone to gibberellic acid. Aleurone isolated in this way, after 3 days treatment with gibberellic acid, contained between 50 and 60% as much α-amylase and β-glucanase as equivalent amounts of untreated fragments supplied with gibberellic acid; the lower figures recorded with the isolated tissue are attributed to damage or losses during separation of the

cells. After 42 hr., we found α-amylase and endo-β-glucanase values for isolated aleurone to be about 40% of those for the whole tissues, although, as is discussed earlier, we do not attribute the difference wholly to losses during processing. No comparison can be made with results obtained by Yomo & Iinuma for proteolytic enzymes, as different methods of assay were used and it is probable that two completely different facets of proteolysis were being explored.

Yomo & Iinuma also measured secretion of hydrolytic enzymes from isolated aleurone: 3 days after application of gibberellic acid to separated aleurone (*i.e.*, 6 days after wetting of the endosperm fragments) they found that 28% of the total β-glucanase of the aleurone had been secreted to the medium, but only 16% of the α-amylase and 10% of the proteinase. In the short-term experiments of the present series, we have found that only endo-β-glucanase is regularly secreted from isolated aleurone—to the extent of rather less than 20% of the total, in 42 hr.

To what extent the secretion of enzymes may be facilitated by the 3-day incubation in *Trichoderma* enzymes remains uncertain: a limited amount of experience with a "cellulase" from snail gut has suggested that results are not essentially different from those obtained by mechanical separation, but we have been reluctant to use fungal preparations because of the possibility of introducing traces of as yet unrecognized biologically active micro-metabolites.

The question of secretion, as opposed to formation of hydrolytic enzymes by aleurone, presents some facets of interest. During the course of this work, Rowsell & Goad[8] reported that isolated wheat aleurone, treated with gibberellic acid, secreted α-amylase into the medium: their technique for separation differed slightly from that described here, but using our own separation methods, we confirmed that wheat aleurone released 740 I.D.C. units of α-amylase, in 48 hr., compared with a maximum of 48 for barley aleurone—and a more usual figure of zero. It must therefore be concluded that wheat aleurone is fully equipped with the mechanisms for formation and for release of α-amylase, while barley aleurone is somewhat deficient in the mechanism required for enzyme secretion, at least in the early stages of incubation. A slow secretion of α-amylase by isolated aleurone fragments was also reported by Briggs,[2] who detected α-amylase in the medium surrounding the aleurone (prepared by treatment with snail juice) only after 4 days of incubation in gibberellic acid, at 18° C.

Finally, in this connection, Varner & Ram Chandra[10] have also shown that α-amylase can be secreted by isolated aleurone. In their procedure, endosperm was first soaked in water for 3 days and the aleurone was then dissected out and treated with gibberellic acid. After a time lag of 9–12 hr. there was vigorous secretion of α-amylase to the medium and, although secretion ceased after 33 hr., it attained a level of over 40% of the secretion by equivalent amounts of entire endosperm: the difference between the two is believed by Varner to be the result of a nutritional deficiency.

There is, then, a substantial measure of agreement between the results obtained by various workers with isolated aleurone, in spite of the differences in types of barley used, in methods of preparing aleurone and in general experimental techniques. No precise quantitative agreement can be expected, for different samples of grain differ markedly in their rates of response to gibberellic acid. That such differences are found even within a variety is apparent from the fact that one sample of Proctor barley required 36 hr. incubation in gibberellic acid before secretion of α-amylase took place from endosperm slices, while a second sample, maintained under similar conditions, responded actively in half that time.

Time course of development of enzymes.—It is generally assumed that, before starch granules in the endosperm can be attacked by amylolytic enzymes, the carbohydrates of the wall must be at least partially degraded, and it is reassuring to find that one of the cytases, *viz.*, endo-β-glucanase, is indeed mobilized in the germinating grain and in gibberellin-treated slices, before the initiation of α-amylase activity. This emphasis on modification of the walls of the starchy endosperm as a necessary prelude to movement of amylase centripetally through the grain conveniently ignores one problem: how does the macromolecule of amylase or, indeed, of any other enzyme penetrate the jungle of microfibrils which constitute the aleurone wall? The cytoplasmic strands, or plasmadesmata, which traverse the aleurone walls (but which

have not been detected in the starchy endosperm) may be of significance in this connection, but a second problem remains: how are the enzymes secreted from the living cell through the cytoplasmic membranes? It is hoped that answers to both these questions may be found eventually by application of techniques of electron microscopy[4] in conjunction with biochemical methods.

Consider first the hydrolases which attack the macromolecules of β-glucan, protein and starch. There appears to be, in barley, a tidy series of events, in the following order: hydration of aleurone, response to gibberellic acid, progressive formation of endo-β-glucanase, then of α-amylase and then of protease, followed, presumably, by hydrolysis of the appropriate substrates. It was earlier suggested[5] that the explosive release of hydrolytic enzymes in response to gibberellic acid was consistant with a gibberellin-mediated release of these enzymes from lysosome-like organelles of the type frequently reported to be present in mammalian secretory tissue. This hypothesis, at least in the simple form first postulated, now seems to be untenable, for two reasons: (*a*) lysosomes would be expected to disgorge all their hydrolytic enzymes simultaneously, and this, manifestly, does not happen in barley or in *Bromus*, and (*b*) fractionation of barley by methods reputed to be adequate for separation of lysosomes has failed to yield any organelles which respond to gibberellic acid by releasing hydrolytic enzymes.

The fourth enzyme studied in the present work—acid phosphatase—is, in animals, a characteristic lysosome enzyme. Results obtained with this enzyme in germinating grain and in endosperm slices are difficult to interpret, because of the high value recorded for phosphatase in untreated, ungerminated endosperm, but the results with isolated aleurone (Fig. 4) are of interest. The untreated aleurone appeared to contain only traces of phosphatase, though values for activity of the enzyme began to rise soon after introduction of gibberellic acid, giving a fivefold increase in 30 hr. The significance of this active phosphatase, in relation to respiratory requirements for inorganic phosphate, and in relation to nucleic acid metabolism, may be considerable. Although the substrate used in this set of experiments—*p*-nitrophenyl phosphate—is not a "natural" one, other work (unpublished) has shown that phytase activity also increases, in endosperm slices treated with gibberellic acid.

In conclusion, it can be said that we now know with certainty which tissue of the grain responds to gibberellic acid; we know that untreated whole grain forms hydrolytic enzymes in the same sequence as gibberellin-treated endosperm slices; and we know that seeds of two unrelated grasses react very similarly to application of gibberellic acid. We now need to explore, in some detail, metabolic changes taking place during the period when, in presence of water, the aleurone increases in its capacity to respond to gibberellic acid; we also require to know something of the events which occur in the aleurone immediately after the introduction of gibberellin and which lead to formation of endo-β-glucanase and other hydrolytic enzymes.

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THE EMBRYO AS AN ACTIVATOR OF GIBBERELLIC-ACID-INDUCED α-AMYLASE

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Evidence is presented which suggests that, in addition to gibberellic acid, a second factor produced by the embryo is involved in the formation of α-amylase by the aleurone of intact barley grains.

Introduction

It has been shown that, although isolated barley aleurone is able to respond to added gibberellic acid by producing substantial amounts of hydrolytic enzymes, secretion of these enzymes into the surrounding medium is very much less than is observed when the adjoining starch-containing cells of the endosperm are still present.[2] It must therefore be concluded that the storage tissue has some contribution to give to the system

aleurone + gibberellic acid → secretion of hydrolytic enzymes.

It is now generally believed[2] that the embryo plays a subsidiary, though important, part in the synthesis of hydrolytic enzymes, one of its major functions in relation to modification being to supply a hormone which activates the aleurone in a manner similar to that observed when gibberellic acid is added to embryo-free corns. However, just as the starch-containing cells of the endosperm exert some influence on the aleurone, so also it is possible that the embryo may have an effect on the aleurone, additional to the production of gibberellin-like material. Experiments have therefore been carried out to determine whether the embryo has any significant influence on the subsequent reactions of endosperm slices exposed to gibberellic acid.

Experimental

Barley grains were first freed from husk by treatment with sulphuric acid, and gibberellic acid (10^{-5}M) was supplied to endosperm slices which had been treated in one of the following ways:

(*a*) Slices were cut from the dry grain and used directly.

(*b*) Slices were cut from dry grain and then allowed to imbibe water for various periods of time before exposure to gibberellic acid.

(*c*) Intact grains were allowed to imbibe water for various periods of time before removal of the endosperm slices.

All incubations were at 25° C. and for control determinations parallel experiments were included with water in place of gibberellic acid. After incubation for 24 hr. the test slices and the controls were homogenized in the ambient fluid and the homogenates were extracted wlth 1% NaCl. The extracts were assayed for α-amylase activity by the method devised by Briggs.[1]

TABLE I

Production of α-amylase in Endosperm Slices (Proctor, 1963 Barley) in response to Gibberellic Acid

Nature of pre-treatment	Length of pre-treatment (hr.)	α-Amylase formed in response to G.A. (I.D.C. units*)
1. None	0	108
2. Slices + water ..	4	224
3. Intact corns + water	4	225
4. Slices + water ..	12	230
5. Intact corns + water	12	260
6. Slices + water ..	24	206
7. Intact corns + water	24	780

Each results in the mean of two separate experiments.

α-Amylase was not detected in any of the controls (*i.e.*, in slices which had been pre-treated as in 1–7 (column 1) and then incubated in water).

* G.A. = Gibberellic acid; I.D.C. unit = Iodine-Dextrin Colour Unit.[1]

Results obtained with a sample of Proctor barley are given in Table I. With this barley, pre-hydration of cut slices caused a doubling of α-amylolytic activity in the extract; however, when water uptake was allowed to proceed in the presence of the attached embryo, α-amlyase formation in response to gibberellic acid was seven times as great as in material from unimbibed corns similarly treated with gibberellic acid. Clearly, the presence of the embryo has accentuated the ability of the endosperm to respond to addition of the hormone.

TABLE II

PRODUCTION OF α-AMYLASE BY ENDOSPERM SLICES FROM DIFFERENT BARLEY VARIETIES

Variety	α-Amylase formed in response to G.A. (I.D.C. Units*)	
	Slices pre-hydrated	Imbibed intact corns
Proctor	250	1356
Hunter	170	800
Mentor	500	812
Union	200	784
Ymer	181	1037

α-Amylase was not detected in any of the controls.
* G.A. = Gibberellic acid; I.D.C. Unit = Iodine-Dextrin Colour Unit.[1]

Other varieties of barley behaved similarly though not all showed an enhancement of α-amylase production when cut slices were pre-hydrated before addition of gibberellic acid. The results in Table II compare the level of α-amylase production in hydrated slices with that in slices cut from steeped, intact grain, both sets of slices being treated with gibberellic acid. It should be noted that α-amylase could not be detected in any of the controls.

DISCUSSION

Although the effect of the embryo in enhancing the response of the aleurone to gibberellic acid is clear, an explanation of this phenomenon is not immediately apparent. The absence of detectable amounts of α amylase from the controls indicates that, if gibberellic acid (or some material resembling it in physiological action) has been translocated from the embryo during pre-treatment of the intact grain with water, then the amount available in the aleurone is less than the equivalent of 4×10^{-8}M, which was the minimal concentration of gibberellic acid required to elicit a response in this system. As pre-treatment of slices with gibberellic acid at concentrations below this threshold value did not allow any greater subsequent production of α-amylase than pre-treatment with water it would appear that gibberellic acid alone is not able to induce maximal production of α-amylase in aleurone.* Some other factor (which might, of course, be one of the other gibberellins) appears to be produced by the embryo and to be involved in the formation of α-amylase. The nature of this material remains a question for speculation and experiment but it is interesting to note that Ng & Audus,[3] working with oat seedlings, have also postulated that an additional unidentified factor is involved, along with indole acetic acid and gibberellin, in growth stimulation of certain tissues of the young seedling.

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* A similar finding has recently been reported by Petridis *et al.*[4]

THE EMBRYO OF BARLEY IN RELATION TO MODIFICATION OF THE ENDOSPERM

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Differential dissection of the embryo of barley grains, followed by observation of the extent of modification and assessment of α-amylase production in the endosperm, has shown that the various organs of the embryo make the following different contributions to modification. The isolated scutellum has a very limited ability to produce α-amylase and its powers of enzyme secretion are largely restricted to the peripheral region where aleurone cells are present. The stimulus which initiates enzyme formation in the aleurone is largely derived from the nodal region of the embryo, especially from the base of the node where subsidiary rootlets are formed; the scutellum apparently lacks the means of producing this stimulus. Translocation of the enzyme-inducing hormone from embryo to aleurone takes place through the apical half of the scutellum in which vascular tissue develops; this preferred route of translocation partially accounts for the asymmetric pattern of modification normally observed. Removal of the first foliar leaf from within the coleoptile allows increased production of α-amylase in the endosperm: this leaf can be regarded as a natural consumer of gibberellin.

Introduction

Much of the recent work reported on the physiology of malting has been concerned with the sequence of events leading to secretion of hydrolytic enzymes, notably α-amylase, by the aleurone layer of the endosperm.[3,14,20,22] The role of gibberellic acid in catalysing the synthesis of these hydrolytic enzymes has been established beyond reasonable doubt and Varner's demonstration[22,23] that α-amylase which is formed by aleurone in an environment containing labelled amino acids and added gibberellin is labelled within the protein molecule and not merely at the terminal positions of polypeptide chains adds weight to the earlier suggestion[3] that production of α-amylase is a *de novo* synthesis of the enzyme. In the absence of gibberellin, protein is synthesized by the aleurone from the labelled amino acids supplied,[23] but only in presence of gibberellin is the protein endowed with α-amylolytic activity. The effects of certain metabolic inhibitors, such as actinomycin D and puromycin, are consistent with the hypothesis that when gibberellic acid is added to aleurone it affects the DNA-controlled synthesis of specific RNA molecules which in turn direct the synthesis of enzymically-active proteins. Suggestions have been made that gibberellins may function in the de-repression of genes which control the formation of the hydrolytic enzymes in germinating barley,[23] and work carried out with oats[18] has yielded results which are essentially similar to those reported for barley.

Concurrently with this body of work on the influence of added gibberellins on isolated fragments of endosperm, has come the recognition of gibberellins as natural constituents of cereal grains. Gibberellic acid itself has been recorded from immature samples of barley[10] and recently, Radley[21] has demonstrated the presence of two further gibberellin-like substances in barley. The obvious deduction which can be made from these two different types of result is that naturally-occurring gibberellins perform the same functions as exogenously-supplied gibberellic acid in malting or in laboratory experiments with tissue slices, and that gibberellins are responsible for initiating the very rapid increase in the activity of hydrolytic enzymes which takes place on the second day on the malting floor.

In drawing these conclusions, some fairly reasonable assumptions are made. Thus, it is presumed that, since the embryo is at least as efficient as added gibberellin in inducing enzyme formation,[13] it is the embryo which is the site of gibberellin production in the grain. This assumption is not wholly unsupported as it has been possible, using thin-layer chromatography, to separate material from excised embryos which has physical and biological properties similar to those of authentic gibberellic acid.[7] The endosperm, however, has proved most intractable as starting material for extracting gibberellins. Further supporting evidence for the embryo as a source of gibberellins comes from early work by Yomo[24] who showed that material which was secreted by barley embryos grown in culture was able to induce amylase formation in embryo-free endosperm. This substance has not apparently been chemically characterized, or indeed, subjected to biological tests for gibberellin activity apart from its effects on barley, though it is believed by Yomo to be a gibberellin.

The embryo of a cereal grain is a complex structure and interpretation of the nature of different regions of the embryo is still a controversial matter (see, *e.g.*, Brown[5]). To assign the task of gibberellin production loosely to the embryo is to leave open a very wide field indeed; with suitable experimental methods, it should be possible to locate this activity in discrete organs of what is, after all, an entire miniature plant. One part of the embryo, the scutellum, is certainly due for a reappraisal of function. This arises from the fact that it is now realized that the aleurone may be responsible for the production of approximately 90% of the α-amylase[3] and β-glucanase[13] during malting. The role of the scutellum as a secretory tissue which is directly responsible for controlling modification is thus very much less than was originally believed[4] and some assessment of the nature and possible extent of the contribution of this relatively massive organ to germination and early seeding growth is therefore desirable.

It seems probable, then, that a gibberellin-like factor is translocated from a site of production in the embryo to a locus of response in the aleroune. There is a time lag of 10–12 hr. between the initiation of enzyme activity in, respectively, slices of endosperm supplied with gibberellic acid and entire grains germinating without additives[13] and this lag is of similar duration for the enzymes β-glucanase, α-amylase and proteinase, which appear successively in that order. It would be an oversimplification to assume that 12 hr. is the length of time needed for movement of stimulating material from embryo to aleurone because hydration of the tissue, which has a marked effect on ability to respond to gibberellic acid,[12] would be expected to proceed at different rates in slices with exposed surfaces and in intact grain covered by a cuticularized testa; none the less, this interval of 12 hr. must represent a maximum time for accommodating the transport of gibberellin to its site of action.

The work reported here has been designed to assess the part played by the embryo in relation to the appearance of hydrolytic enzymes in the adjoining endosperm. It is provisionally accepted that an endogenous gibberellin is involved in stimulating the chain of events which lead to hydrolysis of endospermic reserves: what has been examined is the contributions made by the different organs of the embryo to modification and the path taken by the postulated gibberellin from embryo to aleurone.

Morphology and Anatomy of the Embryo

Surprisingly, few photomicrographs or modern descriptive accounts of the structure of the embryo of barley, appear to be readily available in the botanical literature, though other cereals,[16] particularly wheat and maize, are well documented. As a preliminary, therefore, sections of embryos from whole corns of Proctor barley were cut, freehand or on a freezing microtome or after embedding in wax, and examined after staining with haematoxylin and eosin. A median longitudinal section from ungerminated grain is shown in Fig. 1. Four structurally distinct regions can be seen: (i) the root system with a primary root covered by the coleorhiza and one of the subsidiary root initials; (ii) the acrospire, which includes the coleoptile and, inside this cylindrical organ, the stem apex and leaf primordia; (iii) the nodal region between root and shoot and, laterally (iv) the scutellum, in which a clear distinction can be drawn between the superficial single layer of epithelial cells and the main bulk of the organ.

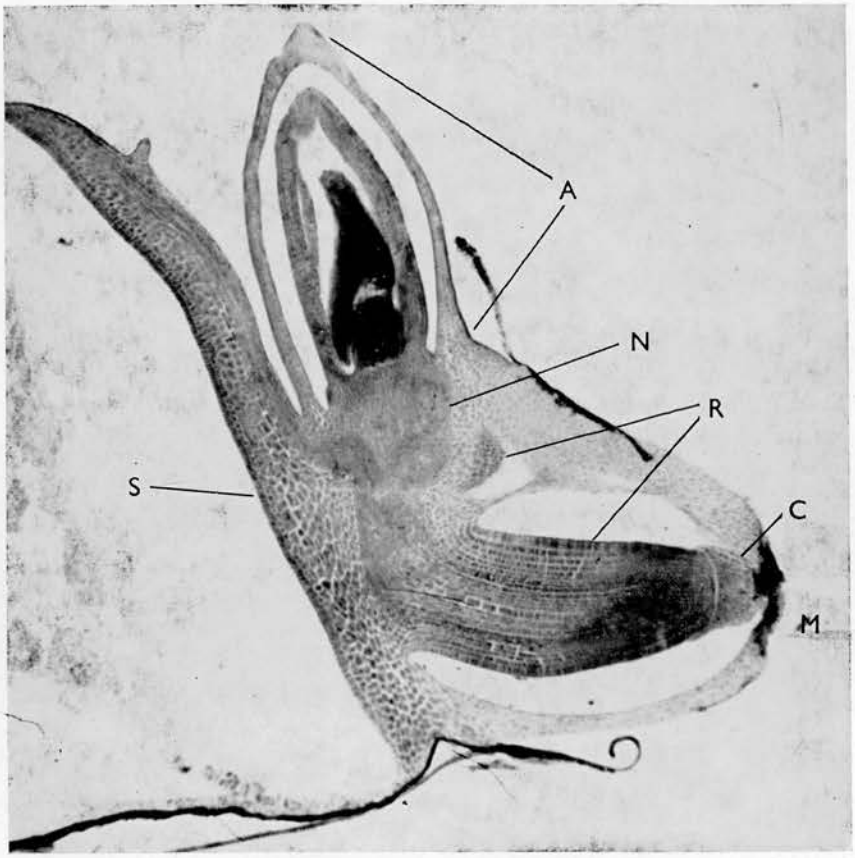

Fig. 1.—Median longitudinal section of embryo from soaked grain (×25).

M—Micropyle.
C—Coleorhiza.
R—Rootlet.
N—Nodal region.
A—Acrospire consisting of coleoptile and enclosed leaf primordia.
S—Scutellum.

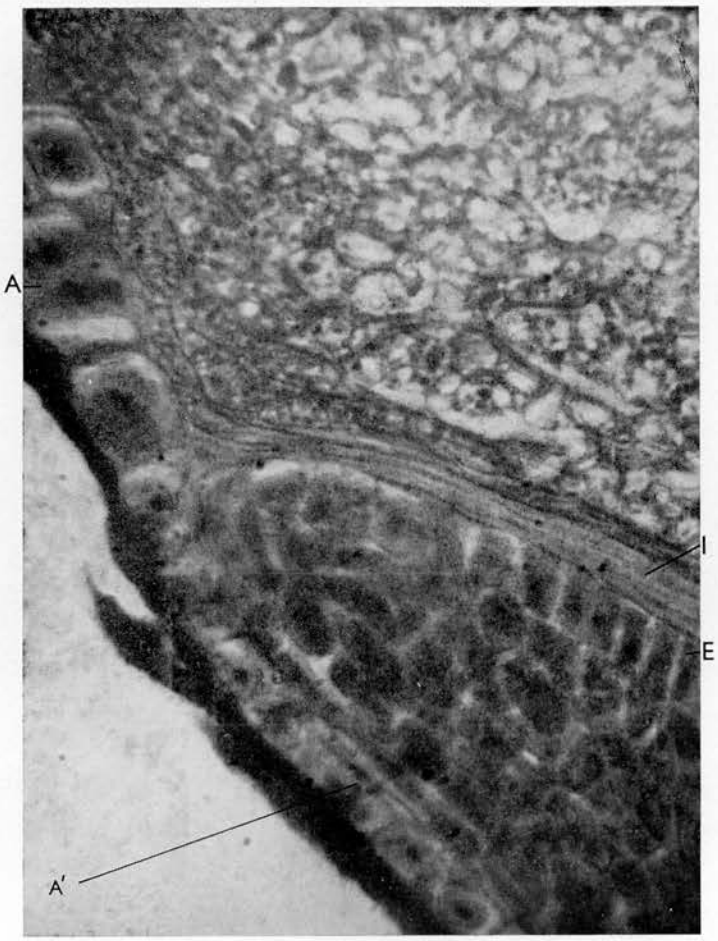

Fig. 3.—Photomicrograph of basal region of scutellum and adjoining endosperm.

A—Aleurone at periphery of endosperm.
A¹—Aleurone at edge of scutellum.
E—Epithelial layer of scutellum.
I—Intermediate layer of endosperm cell walls.

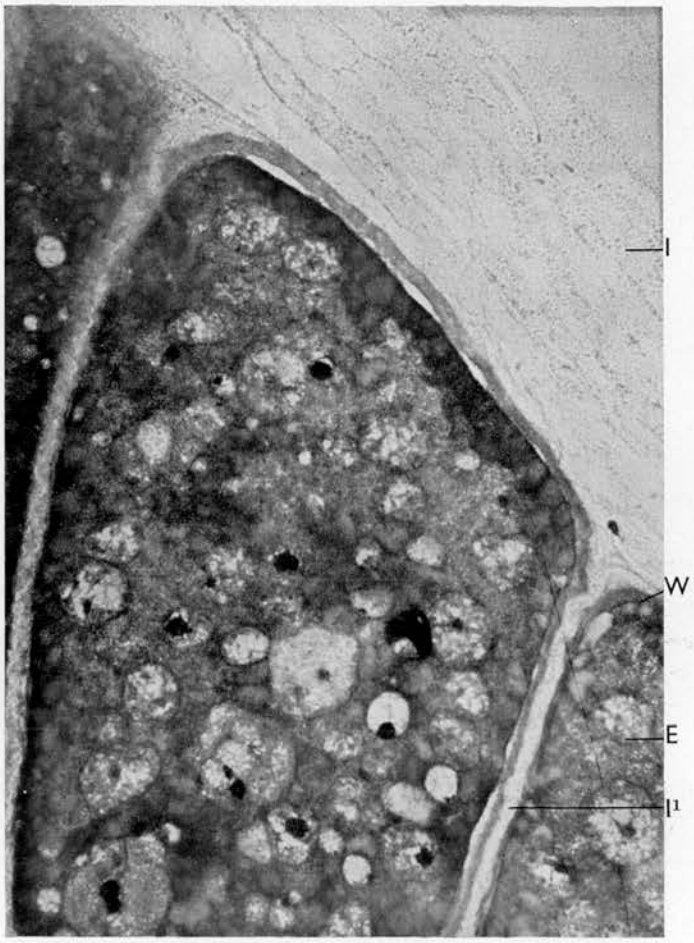

Fig. 4.—Electron micrograph of epithelial layer of scutellum of ungerminated grain.

E—Cell of epithelium.
I, I1—Intermediate layer.
W—Wall of epithelial cell.

In the ungerminated grain the body of the scutellum consists of irregular non-vacuolated parenchyma like cells which lack distinguishing features and show no differentiation; later, cells in the centre of the scutellum are aligned to form a vascular trace. This strand is directed from the node towards the apex of the scutellum, *i.e.*, that part of the scutellum which abuts on the non-furrowed side of the grain. A view taken in one plane only can be misleading and, in order to show the distribution of vascular material more precisely, entire excised scutella were cleared with 5% potassium hydroxide in 80% chloral hydrate and stained with basic fuchsin. The distribution of the lignified tissue at 48 hr. within the scutellum is shown diagrammatically in Fig. 2. The vascular tissues branch throughout the apical region but there is no vascular supply to the part of the scutellum which adjoins the furrow.

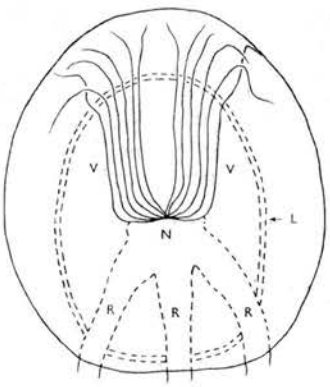

Fig. 2.—Diagram of entire scutellum, stained to show lignification. Grain grown 48 hr.
V—Vascular traces leading from node (N) to apex of scutellum.
R—Vascular supply in rootlets.
L—Line of lignin deposited on cell walls.

We are not anxious to engage in the controversy relating to the interpretation of different parts of the embryo and we propose to follow the suggestions of Brown[5] on this matter: we therefore equate the scutellum with a single cotyledon, we regard the coleoptile as an embryonic organ *sui generis* and the coleorhiza as the residual base of the proembryo, in which the primary root differentiates endogenously, and we treat the nodal region between root and shoot as a single connective structure. This nodal region includes the mesocotyl, *i.e.*, the region between the coleoptile node and the scutellar node.

Two areas of the endosperm which are intimately associated with the embryo present points of considerable interest. First (Fig. 3) there is the aleurone which overlaps the scutellum as a single layer of cells, investing the lower margin of the scutellum to a depth of approximately 500 μ.; it does not, as one report[6] suggests, separate the embryo from the endosperm. The presence of this remnant of aleurone makes it probable that, when embryos are excised, traces of aleurone will be present at the periphery of the scutellum; this is a matter of some importance when the relative contributions of embryo and endosperm are assessed.

A second feature of interest is shown by the part of the starchy endosperm which abuts on the scutellum. Here, as originally noted by Horace Brown,[4] the cellular structure is represented by a series of flattened walls from cells whose original contents have been utilized by the developing embryo. This material, which we term the intermediate layer, is solubilized during malting. High power or, better, electron microscopy of the junction between scutellar epithelium and intermediate layer (Fig. 4) shows that the intermediate material extends between the epithelial cells to a distance of approximately 30 μ.

Histochemical and Other Changes Accompanying Germination

To obtain an overall picture of gross changes which take place, particularly in the scutellum, during the early stages of seedling development, some simple histochemical tests were carried out. These included determinations of numbers of starch granules and of lipid bodies in the scutellum and a visual assessment of the extent of lignification. Results shown in Table I are related to the time of incubation of the grain, to the elongation of cells of the

TABLE I

MORPHOLOGICAL CHANGES IN THE EMBRYO IN RELATION TO MODIFICATION OF THE ENDOSPERM

(Grain grown at 25° C.)

Time after wetting grain (hr.)	Acrospire length (cm.)	Coleorhiza root length (cm.)	Vascular differentiation in scutellum*	Fat deposits in scutellum	Starch in scutellum	Length of scutellar epithelium cells (μ.)	Dissolution of intermediate layer
0	0·10	0·05	0	++++	0	30	0
12	0·15	0·20	Cell elongation	++++	0	30	0
20	0·20	0·25	Lignin +	++++	0	30–40	±†
24	0·46	0·40	Lignin ++	+++	+	40–50	+
48	0·96	1·62	Lignin +++	++	++	70–75	++
72	3·40	2·70	Lignin ++++	+	+++	75–80	+++

* + = a trace; ++++ = maximum amount observed.
† at acrospire end only.

scutellar epithelium and to the state of the interemdiate layer. Though the techniques used were very simple (in the first instance iodine-potassium iodide for starch, Sudan III for lipid and phloroglucinol-hydrochloric acid for lignin) they provide a set of results which illustrate important events which take place simultaneously in the embryo.

Observations made by light microscopy (Table I) indicate that starch granules are deposited in the scutellum by 24 hr. This deposition does not appear to depend on supplies of material translocated from the endosperm because a similar synthesis of starch can be observed in embryos grown, in isolation, on water. Electron microscopy showed that starch could readily be detected at 12 hr.; the numbers of granules present per cell are given in Table II. In parallel with the appearance of starch there is a decline in lipid bodies. Lipid may be transformed into starch, as has been shown to occur during the germination of oil-containing seeds. Such a transformation would presumably be dependent on preliminary action of lipase, which is present in ungerminated barley[15] and, if lipid metabolism in barley follows the same pattern as that reported[11] for castor beans, it would also require isocitritase and malate synthetase activity. Determinations of isocitritase, results of which will be reported more fully later, showed that, though no activity could be detected in the scutellum of ungerminated grain, the enzyme was present by 24 hr. and increased over the 72-hr. period examined. Isocitritase, although detected in the axis of the ungerminated grain, declined rapidly during the first 24 hr. growth. The axis contains much less lipid than the scutellum and does not accumulate starch until about 48 hr.

TABLE II

NUMBERS OF STARCH GRANULES SEEN IN SINGLE CELL PROFILES OF SCUTELLUM*

Length of time grown (hr.)	Number of granules (mean + standard deviation)
0	0
12	8 ± 2·3
24	15 ± 4·5
48	24 ± 2·0
72	25 ± 2·3

* Cells examined were in the body of the scutellum, but adjoining the epithelium. Electron microscopic observation.

Lignin is evident in the scutellum at 20 hr., as annular and spiral thickenings typical of xylem vessels, and is restricted to the orientated vascular tracts shown in Fig. 2. Electron microscopy indicates that the characteristic localized wall thickenings are first detected at 12 hr.

Readily detectable metabolic changes are thus taking place in the scutellum before germination can be said to have occurred—

i.e., before the coleorhiza ruptures the testa —and before the dissolution of the intermediate layer appears to have started.

It will be noted from Table I that the intermediate layer began to lose its structural integrity at approximately the same time as the epithelial cells of the scutellum started to elongate. Both processes started at the apex of the scutellum, near the non-furrowed surface of the grain, and both progressed fairly rapidly to the centre and then laterally throughout the scutellum and overlying endosperm. Earlier work[12] has shown that a rise in endo-β-glucanase is detected at 24 hr. and it is reasonable to attribute the dissolution of this collapsed wall material at least partially to the activity of this enzyme. As the intermediate layer (including the matrix of similar material (Fig. 5) which projects into the interstices of the scutellar epithelium) is solubilized, so the free surfaces of the epithelial cells change in shape from flat to rounded; the cells then elongate, eventually reaching nearly three times their original length. Horace Brown,[4] in his original description of the scutellar epithelium and adjoining layer, attributed the dissolution of the cell walls to direct action of enzymes which were secreted by the extending epithelial cells, which he compared to certain secretory cells of animals. It is tempting, when two phenomena occur in close association, spatially or temporarily, to relate these phenomena causally but, as will be shown later, there is in this case no real justification for attributing hydrolytic properties to the epithelium; it is possibly more accurate to say that elongation of epithelial cells can be observed only after the barrier to growth (the intermediate layer) has been removed.

Secretory Capacity of Different Organs of the Embryo

Having obtained a reasonably clear picture of the structure of different organs of the embryo and of the more obvious changes which can be recognized at germination, methods were sought which would give information on the ability of the individual organs to secrete either enzymes or hormones, such as gibberellins, which in turn would induce enzyme production elsewhere. The procedure generally adopted was to cut away appropriate portions of the embryo and then examine the grain for the developmental changes, such as lignin deposition, extension of epithelial cells and dissolution of the intermediate layer, which had been found to be characteristic of germination; the extent of modification of the endosperm was also assessed. This qualitative, essentially observational study was followed by quantitative determinations of the hydrolytic enzyme which has been most fully studied in relation to gibberellin action, *i.e.*, α-amylase.

Treatment of embryos.—The barley, which had been dehusked by treatment wth 50% sulphuric acid, was allowed to imbibe water from moist filter paper for 2 hr., by which time the embryo was sufficiently swollen to make it possible to excise the required organs with reasonable accuracy, using a small sterile knife. The status of the intermediate layer and the scutellar epithelium was examined in longitudinal sections, the development of vascular tissue was recorded from cleared preparations of entire scutella and endosperm modification was assessed visually on half corns after 72 hr. To make this assessment, the half-corns were vigorously washed and the degree of solubilization of cell contents could be judged in relation to control corns which had been

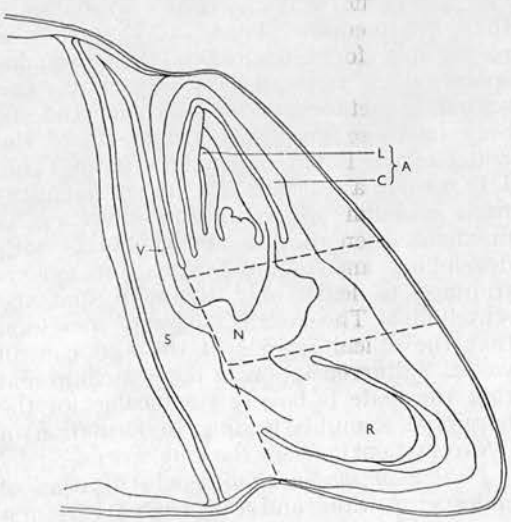

Fig. 5.—Diagram of L.S. of embryo to show incisions required to isolate portions of embryo.
R—Root + coleorhiza.
N—Nodal region.
A—Acrospire, with C—coleoptile and L—foliar leaves.
S—Scutellum, with V—vascular trace.
Lines of incision – – – – –.

TABLE III

EFFECT OF EXCISION ON DIFFERENTIATION IN EMBRYO AND MODIFICATION OF ENDOSPERM

(Scutellum left in position in all treatments)

	Parts of axis removed	Parts of axis left	Vascular differentiation in scutellum*	Elongation of scutellar epithelium cells	Modification of endosperm
A.	Acrospire + Node + Shoot	None	0	0	0
B.	Acrospire + Node	Roots	++	+	+
	Roots + Node	Acrospire	++	+	+
	Node	Roots + Acrospire	+++	0	+
C.	Acrospire	Node + Roots	++	+++	+++
	Roots	Node + Acrospire	++	+++	++
	Roots + Acrospire	Node	0	++	++
D.	None (Control)	Acrospire + Node + Roots	++++	++++	++++

* + = a trace; ++++ = maximum observed.

grown for the same period. The incisions required in preparing embryos are shown in Fig. 5 and Table III gives results of these treatments.

It is clear that the scutellum alone (A in Table III) is unable to accomplish the metabolic changes required to form its own vascular tissue; it is also unable to solubilize the intermediate layer. Whatever is responsible for modification of the endosperm does not arise wholly from the scutellum metabolizing in isolation. Indeed, only in those grain treatments where the nodal region is left undamaged (C in Table III) is there a reasonable degree of modification; vascular differentiation, however, is maximal when rootlets and shoot are both developing and minimal when the axis is trimmed to leave only the node and the scutellum. The results suggest, therefore, that the apical regions of the axis control vascular differentiation in the scutellum and that the node is largely responsible for the hormonal stimulus leading to formation of hydrolytic enzymes in the endosperm.

Pattern of modification.—Solubilization of endosperm walls and cell contents is first seen near the acrospire; later it spreads laterally and upwards through the grain. This asymmetric pattern of wall dissolution has been variously explained as due to thinner walls in the region first attacked,[4] to less dense packing of starch granules, so allowing more rapid diffusion of enzymes in the area seen to be affected[17] and to poor development of aleurone in the furrow, which overlies the part of the endosperm which is slower to modify.[14] Each of these suggestions may have an element of truth in it, but possibly of more basic importance is the architecture of the embryo. To explore this possibility, two series of dissections were performed.

In the first, the region of the scutellum containing the potential vascular system was excised without damaging the node and the grains were then incubated for 72 hr. and examined. It was found that the pattern of advancing modification in the endosperm altered; the rate of modification was diminished and the intermediate layer near the furrow was the first to undergo dissolution. As it has already been shown that the scutellum does not directly contribute to modification it may be that removal of part of the scutellum has removed the preferred route of translocation of a hormone. In the second type of experiment the embryo was excised, inverted and re-affixed on the endosperm by small sterile pins so that the vascularized region was directed towards the furrow. Grains treated in this way modified much more rapidly near the furrow than in any other part. These two sets of observations suggest that the vascular system of the scutellum may be concerned in the transport of the hormone associated with hydrolysis in the endosperm.

Formation of α-amylase in grain with damaged embryos.—Portions of embryos were removed in the manner described above (see Fig. 5), the grains were incubated for 42 hr. and endosperm + scutellum were then removed from 6 grains and assayed by Briggs' method[2] for α-amylase. To make some allowance for the effects of wounding—and these may be considerable—one series of treatments involved simple incisions through the axis from coleoptile tip to the end of the coleorhiza or horizontally at the node. A second additional treatment consisted of slitting open the coleoptile and cutting out the enclosed first leaf. This was a manoeuvre of some delicacy and its success had to be judged at the end of the growth period; failure was frequent and it was necessary to treat about thirty grains and to select for analysis ten on which the operation had been successful. Results of the determinations of α-amylase are given in Table IV.

Clearly, wounding has resulted in considerable impairment of ability to induce formation of α-amylase (Table IV, 2). However, when the effect of wounding is allowed for, the results obtained in grains from which the whole acrospire (Coleoptile + foliar shoot) has been removed (Table IV, 4) are only just significantly different from the control; on the other hand, removal of the nodal region or of all the root initials (Table IV, 5) causes a highly significant impairment in the ability of a damaged grain to form α-amylase. As was previously noted, removal of the whole axis, leaving only the scutellum (Table IV, 3) has a drastic effect on enzyme formation. These results are in general agreement with those suggested by visual inspection of degree of modification (Table III). The final figure quoted (Table IV, 6) is of particular interest. As a cut had to be made in the coleoptile to allow removal of the young leaf this grain comes in the category of "damaged"; nevertheless, there was a highly significant increase in amylase production in comparison not only with the relevant control (Table IV, 2a), but also in relation to the results obtained with intact grain. One of the accepted biological tests for gibberellin involves measuring extension growth of the first leaf of cereals[1] and it must therefore be accepted that endogenous gibberellin is essential to the growth of the organ. When this "sink" for gibberellin is removed, then, presumably, all the available hormone is diverted along the scutellum to the aleurone where it induces synthesis of α-amylase and other hydrolytic enzymes. If this is so, then the results of treatment 4 in Table IV require further consideration. Removal of the complete acrospire (coleoptile + enclosed leaf) did not cause a fall in enzyme

TABLE IV

DEVELOPMENT OF α-AMYLASE IN GRAINS FROM WHICH PARTS OF THE EMBRYO HAVE BEEN REMOVED

Grain treatment	Condition of embryo	α-amylase at 42 hr.*
1. None	Intact	117 ± 12
2. Longitudinal cut down axis	Damaged	66 ± 7
2b. Deep cut at node	Damaged	55 ± 3
2c. Coleoptile tip and Coleorhiza removed	Scutellum and most of axis present	76 ± 16
3. Axis removed	Scutellum only present	10 ± 0·1
4. Acrospire removed	Scutellum, base of nodal region and roots present	46 ± 5
5a. Nodal region largely removed	Scutellum, acrospire, roots and vestiges of apex and base of node present	33 ± 7
5b. Roots and base of node removed	Scutellum, acrospire and vestige of apex of node present	19 ± 7
6. Foliar leaf removed from interior of acrospire	Scutellum, coleoptile, nodal region and roots present	165 ± 16

* Iodine-dextrin-colour units[2] from 6 grains.

TABLE V

α-AMYLASE IN CENTRAL CORES AND PERIPHERAL RINGS OF SCUTELLUM

	Origin of experimental material*	α-Amylase (arbitrary units) (mean and standard deviation)	
		Cores	Rings
A.	Whole grain, grown 48 hr.	2·5 ± 0·2	11·2 ± 0·6
	Excised embryos, grown 48 hr.	0·6 ± 0·1	8·9 ± 0·5
	Excised embryos, grown 48 hr. + 10^{-5}M G.A.	0·7 ± 0·2	10·5 ± 0·5
B.	Scutellum, excised after 2 hr. and incubated 46 hr. in water	2·6 ± 0·3	5·1 ± 0·5
	Scutellum, excised after 2 hr. and incubated 46 hr. in 10^{-5}M G.A.	2·4 ± 0·2	7·6 ± 0·7
C.	Scutellum, excised after 2 hr., and incubated 46 hr. in 10^{-11}M I.A.A.	2·6†	14·8 ± 0·06
	Scutellum, excised after 2 hr. and incubated 46 hr. in 10^{-5}M G.A. + 11^{-11}M I.A.A.	2·5†	20·1 ± 1·0

* G.A. = Gibberellic acid; I.A.A. = Indolyl acetic acid.
† One determination only.

production to a level significantly below that of the most seriously damaged grain; on the other hand, there was no obvious increase in activity as might have been expected in the absence of the leaf. It is possible, therefore, that the region of attachment of the coleoptile, which was also removed, may play some part in inducing enzyme formation.

α-Amylase production by excised embryos.—As anatomical studies had shown that aleurone is intimately associated with the margin of the scutellum (Fig. 3) it seemed desirable to examine the efficiency of aleurone-free and aleurone-contaminated parts of the embryo as producers of α-amylase. To do this, a miniature stainless-steel borer was constructed; this instrument stamped out 1-mm. cores from the centre of the scutellum leaving rings of scutellar tissue contaminated with aleurone. Samples of equal weight were taken (i) from whole grain, (ii) from excised embryos and (iii) from scutella which had been excised after a 2-hr. steep. For (iii), rings and cores were incubated, either in water or in 10^{-5}M gibberellic acid. α-Amylase was then determined by Briggs' method.[1] Results are given in Table V. Clearly, added gibberellic acid has no major effect on the amylase-secreting potential of the embryo (Table V, A); equally obvious is the fact that the peripheral rings, containing aleurone, are very much more active in enzyme secretion than are the central cores.

During the development of this method, it was found that the excised scutella became flaccid and showed a tendency to fragment when they were submerged; this was eventually overcome by agitation during incubation but, among methods examined with a view to maintaining turgor, the effects of indolyl acetic acid were explored. At very low concentrations (10^{-11}M) indolyl acetic acid prevented flaccidity, and it also caused a significant enhancement in secretion of α-amylase by the scutellar rings (Table V, C). Conversely, application of the anti-auxin, tri-iodo-benzoic acid, resulted in a decline in enzyme secretion. This effect is being investigated more fully, and no explanation can as yet be advanced to account for it.

DISCUSSION

Functions of the scutellum.—The results presented above show that 6–12 hr. after the grain has started to imbibe water, the scutellum is metabolically highly active. Lipid bodies are being degraded, starch granules are forming, cells in the potential vascular tract are elongating and lignin deposition has begun. The scutellum is not, however, a major site of α-amylase production, and of the small amounts of this enzyme which might be attributed to scutellar activity, some 80% is formed in the region contaminated with aleurone. Nor is the scutellum of importance in the production of enzymes which disrupt the

intermediate layer and the walls of the central endosperm. In isolation from the axis it is unable to make any impression on the intermediate layer and it seems highly probable that almost all of the machinery for mobilizing endosperm reserves is operated through the aleurone. The scutellum provides a channel for transporting the appropriate hormones and its vascular system may facilitate rapid movement of gibberellic acid, directing it to the region where modification can be seen to commence. Transport of gibberellic acid is not restricted to vascular tissue, and when the vascular tissue was removed, diffusion of stimulatory material through the parenchyma of the base of the scutellum was still possible. It should not be inferred that xylem is the channel necessarily involved in transport to the apex of the scutellum; though phloem was not detected it may also have been present, and it would certainly be expected to function in sugar translocation from endosperm to axis.

A second important function of the scutellum is undoubtedly absorption of endosperm reserves. Edelman *et al.*[8] have shown, quite convincingly, that the scutellum of barley is involved in the transformation of glucose to sucrose, which is then presumably translocated to the axis. The elongation of the epithelial cells, which appears to be possible only after the intermediate layer has been removed, provides an approximately 8-fold increase in absorptive surface at a time when sugars must be accumulating in the endosperm. That the epithelium is potentially highly active is suggested by the large numbers of mitochondria which can be detected therein; an active transport mechanism for absorption of sugar seems not unlikely. The body of the scutellum differs from the epithelium in containing relatively few mitochondria and many plastids. Niewdrop & Buys[29] associate the high content of various metabolic organelles in the scutellar epithelium with its presumed secretory activities: we are convinced that neither α-amylase nor gibberellin is secreted extensively by this tissue. We consider that the epithelium functions as an absorptive layer, and the body of the scutellum acts, like many other cotyledons, as a storage organ and, additionally, provides a vascular system which functions in two-way transport.

The method by which lignification is accomplished in the vessels of the scutellum is of interest. It has been found in the present study that deposition of lignin is associated with the presence of the root and shoot apices in the embryo. It is generally accepted that indolyl acetic acid is produced in apical meristems and that indolyl acetic acid is involved in the process of lignification. The possible part played by indolyl acetic acid in barley germination will be explored further, both in relation to lignification and in connection with enzyme synthesis in the aleurone.

Gibberellin formation in the embryo.— Results obtained by methods which involve surgical treatment of living tissues are always open to criticism, as the damage caused can set in motion various reactions which are not typical of the intact organism. In the work described it was obvious that brown material was being deposited on the edges of the cut surfaces within a few hours of the beginning of the experiments and the metabolic changes induced by wounding are reflected in the 40% reduction in α-amylase secretion noted in material which had been minimally disturbed. However, no better method for exploring the site of hormone production suggested itself, and, for the present, the results can only be taken, cautiously, at their face value.

The scutellum alone is apparently unable to secrete gibberellin, a disability which it shares with maize,[9] and it appears that the acrospire and the coleorhiza and established roots are of much less significance in relation to gibberellin production than is the nodal region—the scutellar node and the coleoptile node. Indeed, the foliar leaf enclosed in the coleoptile can be regarded as a consumer of gibberellin for, with this organ removed, α-amylase production was greatly in excess of that found in whole grains with actively developing acrospires. The nodal region is unfortunately difficult to work with: the cells are not regularly orientated and it has only been possible to treat the whole region as one unit, though in other cereals the cotyledonary node, the mesocotyl and the scutellar node may develop differently in different environmental conditions. In barley, these nodal cells all showed essentially similar sub-cellular inclusions, they were well provided with mitochondria and many were potentially meristematic. From the

base of the nodal region rootlets can be regenerated when, for example, the main root system has been killed by resteeping the grain. This basal root-bearing part of the nodal region seems to be of particular importance in gibberellin production.

The importance of this nodal region of the embryo has already been shown, empirically, from quite a different point of view. It will be recalled that in assessing viability by means of tetrazolium salts, a corn is classed as viable for malting, even if the roots fail to stain but it is rejected if the region between roots and coleoptile base remains colourless. Active respiration in this zone is therefore a prerequisite for successful germination—and successful malting. To what extent this requirement for active respiratory sites is linked with the production of gibberellin remains an open question.

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XXI

PRESIDENTIAL ADDRESS—
On Barley

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On Barley

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It is a useful though humbling exercise for a botanist to select any plant with which he claims familiarity, to assemble all the facts known about it and to write a critical account of its structure and its physiology, its biochemistry and its genetics, its cytology and its ecology. What emerges from the results of such a task is not usually a definitive account of a species but rather a whole host of fascinating and challenging unsolved problems—and an appreciation of one's own ignorance. It might be imagined that the major cereal crops would provide exceptions to this state of affairs and that they at least would by now have yielded most of their structural and metabolic secrets to the army of assiduous investigators who have been at work since man first engaged in agriculture—but have they?

Consider, for example, the barley plant. Among the cereals barley has a good claim to be regarded as the first to undergo domestication. Carbonised ears of 6-row and, less plentifully, 2-row barleys in a good state of preservation were recovered from straw-lined pits in the Fayum some sixty miles south-west of Cairo, and are believed (Jackson, 1933) to date from at least 5,000 B.C., but the earliest domestication of *Hordeum* must have antedated these neolithic Egyptian grains very considerably. As

far as Britain is concerned, neolithic man was growing barley by 2,000 B.C.: indeed, to judge from the numbers of cereal grains which have been preserved by accidental baking into pots, it seems that by the middle of the Bronze Age barley accounted for some 90% of all the cereal cultivation in this country. Godwin (1956) has suggested that the Bronze Age in Britain corresponded with the sub-boreal and thus with climatic conditions peculiarly suitable for the growth of barley; certainly, recognisable grains from the Bronze Age have been recorded from the south of England, from the Dean Bridge and Craigentinny areas of Edinburgh and from as far north as Orkney. Unfortunately, the microspores of cereals give no clue to their former frequency, for not only is discrimination between individual cereals impossible, but only in rye is the pollen extensively wind-dispersed.

It is not surprising that, in the course of years, enormous numbers of papers have been written on various aspects of barley and its cultivation. Takahashi (1943) has estimated that up to 1941 over 1,300 articles had been published on barley: there is no sign of this spate subsiding, as can be seen from the fact that the last 10-year index of the *Journal of the Institute of Brewing* devotes 300 entries to this plant alone. This intensive interest in barley is due partly to its economic importance, partly to its suitability for cytogenetic studies and partly to its availability and ease of cultivation in the laboratory, but a major explanation lies, I think, in the fact that the first really critical investigation of the physiology of germination of any seed was carried out by the apprentice brewer, Horace Brown, in the 1880's. Brown naturally used barley as his experimental material and his pioneer work laid such a firm foundation that many subsequent investigators have also turned for experimental material to a plant which had previously proved amenable to scientific study—possibly ignoring the fact that the success of the earlier investigation was attributable to the brilliance of the investigator rather than to the co-operation of the material investigated.

Be that as it may, there is no doubt that barley certainly has a distinguished historical record, and that it has had an excellent "press" in the scientific literature. To what extent can we now say that, as a consequence of all this activity, we understand the barley plant?

TAXONOMY OF BARLEY

In discussing any subject scientifically, it is usual to begin by defining terms, and, for barley, this entails having a brief look at the various cultivars of *Hordeum*. It will come as no great surprise to learn that different taxonomists—for example, Nevski (1941), Åberg (1946), Bergal (1950), and Bowden (1959)—have all sponsored different schemes of classification of *Hordeum,* but fortunately, most are agreed on one point—that all cultivated barleys can be assigned to the section *Cerealia* Ands., all members of which are able to hybridise freely. Bowden, incidentally, taking his stand firmly on the Code of Botanical Nomenclature (Lanjouw *et al.,* 1956) asserts that Sect. *Cerealia* should be called Sect. *Hordeum,* that the type species is *H. vulgare* L., that the 2-rowed cultivars are largely referable to *H. vulgare* subsp. *spontaneum* and the 6-row to *H. vulgare* var. *vulgare* + *agriocrithon* and that no real difficulties exist if all cultivars are dealt with according to the Code for Cultivated Plants (Fletcher *et al.,* 1958).

Most cultivated barleys are self-fertilised and it is probable that the old-established cultivars, especially the so-called "land" varieties, are essentially homozygous. All have 7 pairs of rather large chromosomes and, in contrast to the bread wheats, the cultivated barleys have, in general, remained diploid; nevertheless, despite this unadventurous restraint from polyploidy, the barleys have achieved a wider geographical range—both in latitude and in altitude—than any other cereal. There are no apparent fertility barriers within the *Cerealia* and crosses between *H. spontaneum* and a mutant of *H. hexastichum* (Beaven, 1947) have yielded in F2 and subsequent generations a wonderful range of morphological variants—showing a range of external characteristics which doubtless could be matched by an equal number of less-easily observed biochemical and physiological differences. With this fine assortment of varied characteristics, widely spread over a number of different cultivars, it would seem that, in the hands of the plant breeder, barley has a bright and assured future. Here, however, some reservations must be made. Different types of barley could certainly be produced *ad lib.* by suitable hybridisation and selection—but not until the primary user of the grain, especially the maltster, can state in precise terms what combination of characters he wants in his grain will it be possible to exploit this genetic variability to the full.

THE BARLEY GRAIN

Consider now the barley grain—the part of the plant for which the whole crop is grown. Fertilisation apparently presents no unusual features (Krauss, 1932), and the triple fusion nucleus, the progenitor of the endosperm, initiates rapid synchronous nuclear divisions which begin before the primary fertilisation is quite completed. No cell walls are laid down in the developing endosperm until over a hundred nuclei have accumulated, and then, more or less suddenly, there appears a reticulum of wall-like material, segregating one nucleus (or sometimes two) per "cell". The biophysics of this process seems to be completely unexplored, but it would surely be rather an interesting, though technically difficult, study. The peripheral cells of the endosperm continue in active division and the three outermost layers assume the characteristic configuration of the aleurone layer—thick-walled cells, rich in fats, in protein and in sucrose, and, unlike the central starch-filled cells, containing a highly active lipase system (MacLeod & White, 1962), and at least some of the enzymes associated with aerobic respiration. Starch granules can be detected in the endosperm 3-4 days after fertilisation (Whistler & Spencer, 1958) and accumulation of starch continues in an orderly fashion, first in the distal region of the grain under the furrow, then in the middle of the flanks of the grain, and, last of all, less than three weeks after flowering, in the sub-aleurone layer.

These events can all be observed by the assiduous microscopist, as can the development of the embryo—but a record of development is surely only a statement of facts in search of an explanation. And even the facts for barley are found, if one delves a little more deeply into them, to be rather unusual. Consider first the contents of the endosperm. Accumulation of starch from primary photosynthetic products is accepted as normal in plants, but in barley the leaves and internodes accumulate sucrose and fructosans preferentially, and starch is formed only in exceptionally "good" years (Yemm, 1935). Fructosans are certainly still present in the grain (MacLeod, 1953), but the predominant activity of the triploid cells of the developing endosperm is in the direction of starch synthesis. What occasions this change in balance, presumably towards enhanced activity of starch phosphorylase? We do not know, but herein surely lies a fascinating problem ripe for the joint attention of cytologist and

biochemist. Moreover, the origin of the endospermic starch is unexpected. Up till quite recently physiologists would have agreed that carbohydrate reserves in seeds accumulated from sugars synthesised in the leaves and translocated upwards in the phloem at breathtaking speed: this may indeed be true for many plants but, as Porter *et al.* (1950) and, more recently, Buttrose & May (1959) have shown, the activity of the ears themselves may contribute the greater part of the starch in the endosperm.

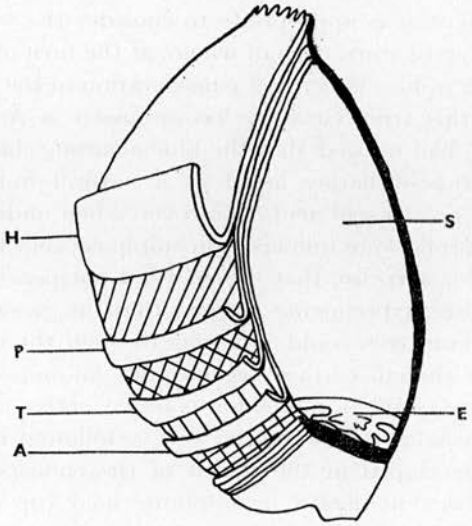

FIG. 1.—Outer coverings of barley grain. H, Husk; P, Pericarp; T, Testa; A, Aleurone and remains of nucellus; E, Embryo; S, Starchy Endosperm.

During this period of active starch accumulation within the developing grain, spectacular changes are also taking place in the surrounding pericarp and testa. In Proctor barley, growing in Edinburgh, measurements made at daily intervals showed that the length of the pericarp quadrupled in the first five days after fertilisation, so attaining three-quarters of its length at ripeness. Meanwhile the outer layers of the pericarp disintegrated to expose the middle chlorophyllous tissue, the outer integument degenerated and the inner integument became invested with a thick cuticle-like layer which was especially dense on the outer surface. Adhesion of the glumes to the kernel began about nine days after fertilisation and was at first most obvious on the ventral (palear)

surface, but by three weeks after anthesis both lemma and palea were attached fairly firmly to the caryopsis. The "gum" which fixes the husk to the caryopsis could presumably be derived from the disintegrating outer layers of the pericarp, but the origin of this material is somewhat speculative and needs further investigation. Certainly, by the time the grain is ripe it is covered in a fearsome array of coats: these are shown diagrammatically in Fig. 1.

THE PERICARP-TESTA.

At this point it is appropriate to consider the work of a trio of brilliant investigators, each of whom, at the turn of the century, devoted much of his energy to a consideration of the barley grain. The first of this triumvirate to be discussed is Adrian Brown. Brown (1907) had noticed that the blue aleurone characteristic of certain varieties of barley acted as a natural indicator which turned red in presence of acid. However, when undamaged corns with blue aleurone were immersed in sulphuric acid Brown found, doubtless to his surprise, that the acid did not penetrate into the aleurone. Later experiments showed that, in general, inorganic salts and certain dyes could not pass through the cuticle of the residual testa though certain organic and un-ionised substances could penetrate readily; iodine, for example, enters the grain with ease and its passage to the interior can be followed by noting the blue colour developed in the starch of the endosperm. Corns which have been immersed in sulphuric acid (up to 50% concentration) imbibe water in the usual fashion and, after the acid and the disintegrated outer tissues have been washed from the seed, the grains will germinate. Brown found that the semi-permeability of the cuticle of the integument was not destroyed by boiling the grains (though naturally they failed to grow after boiling), but semi-permeability was destroyed by either 1% sodium hydroxide or dilute nitric acid. It is interesting to speculate on the chemical nature of a natural material which is altered in some fashion by dilute nitric acid but apparently unaffected by 50% sulphuric acid, and application of modern microchemical methods to this problem might yield interesting results.

Brown's original observation has been confirmed by several workers (e.g. Collins, 1918; Tharp, 1935), and Brown's work has proved most valuable in relation to problems of dormancy in barley. The whole subject of dormancy in seeds is complicated.

Not only are physical restraints imposed by the various coverings investing the embryo, but maturity (whatever that may be) of the tissues of the embryo and the presence or absence of growth inhibitors and stimulants, may all be involved in determining whether or not a seed will germinate. Recently, work by Pollock and his associates (1955) has done something to clarify the nature of the dormancy encountered in barley—and understanding the problems involved is surely the first step towards solving them.

Briefly, Pollock *et al.* have shown that the apparent intensity of post-harvest dormancy in barley may vary according to the amount of water supplied to the corns in the germination test. Thus, in certain samples of barley all corns germinate when only 4 ml. of water is supplied to a petri dish containing 100 grains, but many fail to grow with 8 ml. of water present. This inability to germinate in presence of liberal amounts of water has been termed water-sensitivity. Again, other samples of dormant grain may be indifferent to the precise level of water provided, and fail to germinate at any level of availability of water. Fortunately, both water-sensitive and truly dormant barleys "mature" and, after a few weeks or months of storage, grow satisfactorily in various water régimes. Numerous more or less empirical devices have been recommended in order to secure maximum germination in the large bulks of grain which may have to be malted soon after harvest. Water-sensitivity can obviously be controlled by adjusting the balance of water and oxygen supplied to the steeped corns, while the water-indifferent type of dormancy can be alleviated by hydrogen sulphide. All this is rather confusing, but some slight semblance of order can be established if we reconsider the recorded facts in relation to the various tissues investing the grain.

When husk and pericarp plus testa are removed from the grain, both water-sensitivity and ordinary dormancy are eliminated, but if the husk alone is carefully detached, then water-sensitivity and dormancy persist. This clearly indicates that the pericarp-testa is implicated in both types of barley dormancy. Now, if the grains are steeped in 50% sulphuric acid so that all tissues outside the cuticular layer of the testa are destroyed, the germination of water-sensitive and dormant barleys is complete and successful. The obvious deduction is that the remnants of the pericarp are intimately involved in imposing a restraint on the germination of freshly harvested grain. But why? And how?

It seems moderately certain that availability of oxygen to the tissues of the embryo is a factor of over-riding importance for the germination of most seeds and, if the pericarp of newly harvested barley restricts the passage of oxygen to the embryo, then difficulties in germination could be expected. Pollock (1958) has suggested that active phenol oxidases in the pericarp might consume oxygen and that the improved germination observed in presence of hydrogen sulphide is due to inhibition of these oxidases. Approaching the problem from rather a different point of view, Bishop (1944), who carried out extensive investigations into post-harvest dormancy, has suggested that since the pericarp is exposed to the atmosphere (as the ovary wall) at anthesis, mould spores must settle on its surface and the resulting mycelium, still present when the grain is ripe, would both consume oxygen rapidly and, by virtue of its mucilaginous walls, impede the passage of gases. It may be suggested that these two hypotheses can neatly be combined to give the deduction that phenol oxidases of moulds growing on the pericarp may be influential in barley dormancy. Bishop has also suggested that *Aspergillus,* for example, could ultimately be responsible for overcoming barley dormancy, since its ability to grow in presence of only 16% of water might eventually cause sufficient breakdown of the pericarp to allow entry of gases. There is evidence, too, that barley dormancy is partly a varietal characteristic (Bishop, 1958): surely, with all this wealth of fact and speculation, a planned attack by histologists, mycologists and biochemists could resolve many of the problems posed by the pericarp.

Just how water sensitivity fits into the picture is far from clear. However, working with mature barley, Robert Brown (1943) has shown that excised embryos behave very differently from intact corns in their uptake of water and in their gas exchange: would it not be interesting to extend this work to include measurements on water-sensitive barleys which have been dehusked, then treated with sulphuric acid to remove the pericarp (and abolish water sensitivity) and finally dissected to expose the embryo?

All this discussion of dormancy has ignored the effects of high temperature (40° C.) and low temperature (4° C.), both of which are strongly advocated by different workers as cures for dormancy: both conditions may indeed be effective in eliminating

dormancy. Nor is any attempt being made to discuss the effects of kinetin or gibberellic acid on barley dormancy—and both may be effective in breaking dormancy—for the theoretical background to the action of these growth stimulants is so vague that no useful purpose would be served by adding to the confusion here. Enough has been said about this difficult problem to show something of the complexities which have been uncovered in these apparently insignificant outer layers of a barley caryopsis since the days of Adrian Brown's first observations on semi-permeability. These tissues are perplexing indeed—but consider now the inside of a barley grain, and especially its endosperm.

CARBOHYDRATES OF THE ENDOSPERM.

As a starting point, let us revert to the activities of the nineteenth century brewers, and consider the contributions made by one of the early carbohydrate chemists—Cornelius O'Sullivan. In addition to establishing the fact that sucrose is quantitatively the most important sugar in barley embryos, O'Sullivan (1885) was the first to detect the trisaccharide raffinose in barley and to compare the sugar content of a barley with that of its malt. Although repetition and extension of this early work (see, e.g., James, 1940, MacLeod, *et al.*, 1953) has filled in details of sugar metabolism and added the fructosans to the metabolically important sugars, and Edelman *et al.* (1959) have recently shown the importance of uridine diphosphate glucose in converting starch from the endosperm into sucrose in the scutellum, O'Sullivan's pioneer analyses remain unchallenged. Of even greater interest than his work with sugars was O'Sullivan's demonstration (1882) that two different non-starchy polysaccharides could be extracted from barley grains —materials which could be dissolved in water and precipitated therefrom by various organic solvents to yield long white fibres. O'Sullivan believed that his products were in some way related to starch and he called them α-and β-amylans. These two materials were mere biochemical curiosities for half a century, but recent work by Preece and his collaborators (Preece, 1957) have brought them into the limelight again. These amylans are not really related to starch, but are rather associates of cell walls and are best considered in relation to that unfortunately named group, the hemicelluloses. Indeed, modern techniques involving chromatographic separation of the constituent sugar units after

the polysaccharides have been hydrolysed have shown that O'Sullivan's products (which have fortunately been preserved) are mixtures of some of the extractable hemicelluloses.

These hemicelluloses are of great importance in the germination of a barley corn—or, to be more precise, their successful elimination is important. Germination of barley has been most fully studied in relation to malting, and one of the principal differences between an ungerminated grain of barley and its germinated counterpart (which may loosely be referred to as malt) lies in the virtual disappearance of the cell walls from the endosperm. What then is the constitution of the endosperm cell walls and how are they disposed of? At first sight such a question seems absurdly childish, for every student of elementary botany has been taught that the walls of unlignified tissues are largely made of cellulose and held together by pectic middle lamellae. Now, if this interpretation of wall composition is correct, the germinating barley corn must produce an enzyme capable of digesting cellulose, namely, a cellulase. Writers of review articles on cellulase indeed tend to seize on the observation of the solubilisation of endosperm walls in germinating grass seeds, for, although they can find many authentic records of cellulase activity in fungi and bacteria, grasses appear to provide the only good examples of angiosperms which produce cellulase. But do they?

Let us pursue the question a little further and trace the references to barley cellulase back to the original starting point. Apart from the anatomical observations, which are not in dispute, the only hard fact available is that an extract of malt can hydrolyse acid-solubilised filter paper to an extent of approximately 7% during several days of incubation at 37° C.—surely rather slim evidence for a highly active cellulase in barley. Again, examining the problem from the point of view of the substrate, MacLeod and Napier (1959) took fractions progressively separated from barley by a pearling machine and found, on analysis, that while the husk, the embryo and probably the aleurone layer are well provided with cellulose, the central portion of the endosperm, the pearled grain, gave a yield of only 0·04% of crude cellulose—a total which is easily accounted for by the husk-like tissues invaginated in the furrow. Thus, since there is no cellulose, there is no need to postulate the presence of a true cellulase in barley—a finding, which, though negative, has the merit of bringing

grasses into line with other angiosperms. Nor, apparently, is there any pectin in barley endosperms; indeed, in a related grass seed, *Bromus sterilis,* direct observation of sections shows that the individual endosperm cells are held together mechanically by the aleurone and husk, for they float out as separate entities if the slightest pressure is applied to the cover glass. The traditional concept of plant cell walls as essentially cellulose stuck together by pectic materials is thus far from true of certain grass endosperms, and we must consequently turn to O'Sullivan's peculiar polysaccharides as the major, and indeed, perhaps the only authenticated components of the endosperm cell walls.

During the past decade these hemicellulosic materials have been intensively studied—biochemically by Preece (1957) and structurally by Aspinall *et al.* (1958): they provide the framework of the endosperm , and they can at least partially be digested by the activities of the growing grain, but they are very different indeed from the familiar ingredients of walls not only in their biochemical composition but also, as noted above, in their actual physical means of incorporation into the visible cell walls. However, it can now at least be said that O'Sullivan's polysaccharides can be assigned with some certainty to a position of importance in the barley corn.

METABOLISM OF GERMINATION

O'Sullivan was a great chemist but he had one failing—he was only a chemist. Analysis of plant materials, removed as rapidly and as completely as possible from the living organism, was his *forte,* and for a real appreciation of the barley grain as a living entity we must return at the last—and probably the greatest—of the group working in Burton in the 1880's—Horace Tabbarer Brown.

Half-brother of Adrian and posthumous son of Benjamin Tabberer, Horace was brought up by his stepfather, Edwin Brown, a bank clerk and an enthusiastic amateur naturalist. Horace was apprenticed to a brewer at the age of eighteen and immediately turned his attention to the complex changes involved in the transformation of a grain of barley into malt. He has recorded that his youthful interest in pond life, encouraged by his stepfather, had made him a competent microscopist, but his gifts did not stop at microscopy. Good diagrams of the structure of the grain of

barley were available at that time, but there was no real insight into its metabolism, an omission which rather surprised the young brewer. In his own words: "It has always been a matter of wonder to me that, having gone so far, these observers should not have extended their botanical studies to investigating the physiological meaning of what they saw and drew so well". He himself soon filled this gap.

I would suggest to you that Horace Brown manifested three of the qualities which help to make up the successful botanist: he was an accurate observer, he handled plant material sympathetically, and, above all, he retained through his working life an open and enquiring mind. Although he was never formally trained as a botanist, his work in conjunction with Escombe on the mechanism of stomata revealed a physical law, previously unknown, which explained many of the mysteries of diffusion of gases through small apertures, and his memorandum on the *Germination of Some of the Gramineae* (Brown & Morris, 1890) is a classic.

Many of the facts which he established for the first time are now accepted as commonplace, but they were novel—and spectacular—at that time. He observed the peculiar behaviour of the scutellar epithelium, whose individual cells elongate and protrude into the starchy endosperm, and he noticed the changes in their cytoplasm from granular to pellucid, so reminiscent of secretory cells in the animal kingdom. From observation he proceeded to experiment, and he showed, by means of an elegant surgical technique, that although the isolated scutellar epithelium could digest starch, the residual embryo and even the scutellum without its epithelium could not. He grew excised embryos, removed from steeped corns, on sucrose and other nutrients and found that the scutellum did not secrete α-amylase or cell-wall dissolving enzymes when suitable sugars were supplied to the isolated embryos. He distinguished between the two different amylases—the β-amylase which is present in resting barley and the α-amylase which is synthesised by the scutellar epithelium only when the grain begins to germinate, and he traced the progressive dissolution of cell walls from the region abutting on the scutellum, along the peripheral starchy cells underlying the aleurone to the distal end of the grain. Later, in conjunction with Escombe (1898), Horace Brown showed that the aleurone also

possesses rather feeble cytolytic potentialities, though the experimental techniques required for demonstration of enzymic activity in the aleurone were far from easy.

Since Brown's time, other investigators, notable Mann & Harlan (1916), have attempted to reach a final unequivocal decision on the possible rôle of the aleurone layer, and the balance of opinion at present favours the scutellum as the major producer and disseminator of hydrolytic enzymes, both amylases and cytases, with the aleurone playing a rather minor rôle. Amylolytic action is on the whole easier to detect than is the initial stage of cell wall breakdown, because the actual pitting of the starch granule gives visible evidence of the action of α-amylase. According to Mann & Harlan (1916) this obvious corrosion of starch grains in the sub-aleurone layer of a barley grain is not evidence of any peculiar activity of the aleurone, but merely a physical consequence of the fact that the sub-aleurone layer is the least densely packed with starch grains, which are rather smaller than those in the bulk of the endosperm; it is thus more easily penetrated by solvents and, by analogy, presumably by enzymes such as amylases and cytases. Analogies can be dangerous, and I must confess that I am a little uneasy about the whole question of diffusion of a large molecule like that of α-amylase from cell to cell of the endosperm. Be that as it may, there is no doubt that, as Horace Brown first showed, excised embryos are able to secrete sufficient α-amylase from their scutellar epithelia to digest a substrate of starch granules in semi-solid gelatin if, and only if, these starch granules are from barley or some other cereal: the excised embryos cannot attack granules of, for example, potato starch.

Horace Brown was a pioneer: how extensively have his successors consolidated the advances he made and solved the problems he uncovered? Possibly because of the excellence and comprehensiveness of Brown's work, the germination of barley and the interaction of embryo and endosperm were largely ignored for the next half century, as far as general concepts of germination are concerned. One interesting advance in understanding was made here in Edinburgh by John S. Ford (Ford & Guthrie, 1908). Whereas Horace Brown had interested himself mainly in the secretory amylase produced in the scutellar epithelium and distributed thence throughout the endosperm,

Ford was concerned with the latent (β-) amylase of the grain and with methods suitable for extracting this enzyme. He found that though β-amylase was to some extent soluble in water, it was more fully extracted from ground barley into 1% salt solutions and still more soluble in presence of the proteolytic enzyme, papain. The obvious implication of this last observation is that papain degrades protein and so allows easier extraction of the β-amylase —though this poses the further problem of why it is that β-amylase, a protein itself, is not destroyed by proteolytic action. Unfortunately, as Ford showed, this logical explanation is not the correct, or at least the only one, for boiled papain is almost as effective as active papain in allowing maximum extraction of β-amylase from barley. As far as I know, this peculiar effect of papain has not been fully explained, though there is much wise talk about its potential efficiency in maintaining sulphydryl groups in the β-amylase.

Ford & Guthrie also determined the distribution of β-amylase within the corn and found, very interestingly, that the proportionate concentrations were approximately: embryo, 1 part, central endosperm, 33 parts, peripheral endosperm, 88 parts—a distribution which becomes particularly noteworthy in the light of recent comparisons of starch from barley and from malted barley. Greenwood *et al.* (1959), also working in Edinburgh, have shown that the principal difference between barley starch and malt starch is a shortening of the external chains of some of the amylopectin molecules in the starch granule—a type of structural change which can be attributed only to the activities of β-amylase. The α-amylase secreted from the scutellum has apparently only a very limited effect during the week or so which elapses while the barley is growing into malt. We might do well at this point to recall another of Horace Brown's convictions, namely his firm belief that no progress would be made in understanding germination so long as we adhered to the plan of investigating the grain as a whole. In this particular instance, analysis of the whole grain of germinated barley reveals a significant amount of α-amylase which plays a predominant part in breaking down starch when the grain is ground up in water; 80% of this same α-amylase, however, is restricted to the proximal one-third of the endosperm, abutting on the scutellum (see, e.g., Graesser & Dax, 1946). What macerated tissues can do and what the same living, growing

organised tissues actually *do* do may often be two very different things: plant biochemistry is a fascinating and rewarding pastime, but, as botanists, we must exercise the greatest caution in extrapolating the results often necessarily obtained by *in vitro* study, back into the growing seedling. We can none the less be moderately confident that we now do know a little about the changes in the starch granules of a germinating grain of barley: Horace Brown laid the foundations of our knowledge by asking the crucial questions; Ford revealed the peculiar distribution of β-amylase; Greenwood built on Ford's observations by demonstrating the association, histologically speaking, between the location of β-amylase and the actual attack on starch, and Graesser & Dax in a sense confirmed this by discovering the very limited extent to which α-amylase penetrates from the scutellum to the endosperm cells.

CELL WALL DEGRADATION

As far as starch metabolism is concerned the situation is, at the moment, moderately tidy. What of the endosperm cell walls and their dissolution, also explored initially by Horace Brown? It seems a fair assumption to say that although the materials comprising these walls have been at least partly characterised and much has been established about the changes in the balance of the various cytolytic enzymes, mainly through the work of Preece and his collaborators in Edinburgh (1956, 1958) and Meredith in Winnipeg (1958), it still remains to relate these changes in substrates and in enzymes to the visible alterations taking place in the actual cells of the endosperm. In other words, the biochemists have provided a wealth of data about the extractable materials, but the botanists have not yet integrated this biochemical information to give an overall picture of what is happening to the wall-like reticulum in the slowly crumbling endosperm.

MODERN DEVELOPMENTS

From this brief discussion of events taking place in the endosperm of a barley corn during the few days when it is being transformed into malt it might be thought that the whole story of the past sixty years was one of unassuming, steady—though possibly rather slow—progress in understanding. Such an impression is only partially true. Progress, I suggest, has come not in response

to painstaking analyses, important though those may be, but in direct proportion to the asking of critical, and often very simple, questions. The ability to ask the rewarding question, and the persistance to find an answer to that question play a greater part in scientific advance than is often realised. However, from time to time discoveries in fields remote from our own particular specialities may pose relevant questions for us. Such a discovery which has had a very considerable impact on problems of endosperm digestion by germinating barley is that of gibberellic acid.

Briefly, it can be said that gibberellic acid, applied to the steep liquor in concentrations as low as one part per million of the barley, can speed up the rate of transformation of a barley grain to malt by as much as two days. The grain which has been treated with gibberellic acid produces more α-amylase than the untreated grain, it digests its cell walls more rapidly and it forms much more active proteinase. All these changes may be of considerable importance economically to the maltster, and it is obviously of the greatest interest to the botanist to try to discover how gibberellic acid exerts its profound influence. Ignoring for the moment the question of how gibberellic acid gets into the grain —and Brian (1961) has shown that possibly only 10% of the small amount supplied actually penetrates even as far as the aleurone— the fundamental biochemical action of gibberellic acid is an intriguing problem.

The present indications are that gibberellic acid exerts its influence not through the embryo, but directly through the endosperm. Paleg (1960), for example, has shown that if barley grains are cut transversely a short distance behind the embryo and the scutellum ends are incubated in presence of gibberellic acid, then sugars—mainly maltose—and assorted amylolytic enzymes are liberated into the surrounding medium in response to the presence of gibberellic acid. Paleg has suggested that the embryo in the intact grain may secrete some gibberellic acid-like material into the inert endosperm to activate previously inert precursors of enzymes. This may be so, but we have found that this enhanced sugar secretion by slices of endosperm takes place only if the aleurone is present: pieces of central endosperm lacking aleurone show no response (MacLeod & Millar, 1962). Moreover, endosperm slices also liberate, in presence of gibberellic acid, at least one of the enzymes involved in disintegrating

hemicelluloses. How the gibberellic acid acts remains obscure, but the observation is an exciting one and its implications are tremendous. These relatively new findings have the merit of concentrating attention on the aleurone layer once again.

They also recall to mind Schander's work (1934) with rice and other grass seeds from which a ring of aleurone had been filed off just above the embryo. Dry rice seeds treated in this manner failed to germinate when subsequently provided with optimum conditions for growth, but a short period of steeping of intact grains sufficed to allow an interaction between embryo and aleurone which was effective in promoting subsequent germination of ringed seeds. It is premature to speculate on the possible intervention of the naturally-occurring gibberellins of barley in these interactions between embryo and aleurone, but herein certainly lies a wonderful field for future investigations.

Indeed, there is no dearth of problems related to barley to suit all botanical tastes. The barley plant may have been grown as an agricultural crop for over seven thousand years, the grain may have been malted since prehistoric times, and the barley plant may have suffered as the victim of innumerable laboratory experiments, and yet, I suggest, we have learnt just enough about the germination of the seed to make it at present one of the most fascinating of botanical studies.

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XXII

THE PHYSIOLOGY OF MALTING - A REVIEW

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Factors which are believed to be responsible for dormancy in barley are discussed in relation to the occurrence of this condi-:tion in other seeds. Structural features of the grain, and particularly the constitution of the pericarp-testa and the micro-:pylar area, are important in problems of dormancy, and physiological factors such as inadequate availability of oxygen and, possibly, presence of inhibitors in the embryo, are also relevant. Techniques used to overcome dormancy (including peeling of the grain, addition of hydrogen sulphide and treatment with gibberellic acid) may help to indicate the causes not only of primary dormancy but also of that form of secondary dormancy which is usually described as water sensitivity.

Modification of the endosperm is briefly discussed in relation to the interacting effects of moisture, oxygen supply, temperature and presence of phytohormones.

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INTRODUCTION

Malting, like barley cultivation, depends on exploiting the physiological processes of a growing plant, but maltsters and farmers have very different ends in view. A maltster considers the grain mainly as a potential source of enzymes, fermentable extract and yeast nutrients, malting involves harnessing and then curtailing the potentialities of the embryo for digesting the endosperm, and seedling growth is tolerable only in so far as it renders the endosperm fit for mashing. A farmer, on the other hand, is concerned with the endosperm merely as a source of nutrients for the seedling during the period when it is not self-supporting. Vigorous growth is agronomically desirable but it may be a most uneconomic feature in malting.

There is a considerable amount of information in the botanical literature on the physiology of barley germination and seedling growth, but much of it is orientated tacitly towards the natural process of seedling establishment rather than towards the restrictive procedure of malting, where suppression of growth, without losing certain of the benefits of the metabolic activities of the embryo, is desired. It must be remembered that malting is not a natural process: malting methods may have become what they are by a process akin to organic evolution, starting from agricul-:tural practice and culminating in the procedures adopted at present, but they are now remote from natural growth processes in the field.

It is possible that procedures for preparing grist from barley may, in the future, exploit the grain in a manner which is very different from malting.[27] At present, however, wort production largely depends on malt made in a more or less traditional manner: the physiological phenomena accompanying this process form the subject of this review.

Despite what has been said above, there is one essential stage in malting which is equally important in agricultural practice and in malting - germination of the grain. Germination is defined in a recent monograph[67] as "the consecutive number of steps which causes a quiescent seed, with a low water content, to show a general rise in metabolic activity and to initiate the formation of a seedling". The necessary consecutive steps are as yet poorly defined, though the study of seeds which have exacting requirements (e.g. for low temperature treatment or for critical conditions of illumination[67]) is helping to explore the pattern of change between quiescence and growth. Normally, germination proceeds fairly smoothly in cultivated barley, but the difficulties which are occasionally encountered may be instructive in that they direct attention to phases in germination which usually contribute smoothly to the overall mechanism and are recognized only when they are out of gear. In this review it will be necessary to examine the cause of impediments to germination in seeds other than barley as these may illuminate events which occur,

unrecognized, also in barley.

In the definition of germination cited above it will be noticed that the phenomenon is considered to be complete when seedling initiation is under way. If this is accepted, germina-:tion ends when the coleothiza has ruptured the seed coat (i.e. when the grain has chitted), and subsequent developments should be referred to in different terms. "Seedling growth" is an adequate descriptive phrase if, as in agricultural practice, the events following germination are aimed at producing the next generation of the plant, but in malting it is inappropriate and may obscure the nature of the processes involved in modification. It is suggested that in descriptive work on malting the period after chitting should be referred to simply on a time basis, as hours (or days) from wetting the grain and that rather loose terms such as "third day of germination", should be avoided. After all, if the third, why not the fifty first: with this usage when does germina-:tion terminate?

Modification - Here we are dealing with a concept, rather than with a group of facts which are susceptible of neat definitions. The wave of metabolic activity which passes from the proximal (embryo) end of the endosperm towards the distal apex of the grain and which is characterized by progressive liberation of hydrolytic enzymes from the aleurone is the immediate cause of modification; the result is the characteristic transformation of tough barley corns

into friable malt. Assessment of modification by biting is a traditional method of determining how extensively the walls of the endosperm cells have been degraded; measurements made by sclerometer[99] or murbimeter[24] give more sophisticated and less subjective estimates of the same phenomenon. The changes estimated are in grain texture and they depend largely on changes in the physical nature of the cell walls of the endosperm; indirectly, they reflect the progression of cytolytic activity through the endosperm. Differences in extract obtained from fine- and coarse-ground malt, which give useful assessments of modification,[50] not for individual corns, but as average figures for a large number of grains, depend both on the breakdown which has occurred in the walls and on the results of amylolysis during mashing.

Alterations in carbohydrate balance during malting are not the only transformations which contribute to successful modification. Considerable attention has been paid to a summarized estimate of gross changes in nitrogenous constituents during malting, and the proportion of the malt nitrogen which has been degraded to a permanently soluble form has been referred to as the Index of Modification.[53] The wisdom of implying that the fate of one type of material can adequately reflect the whole range of biochemical transformations which combine to constitute modification may be doubted,[80] especially in view of the fact that procedural changes

in techniques of malting may affect different facets of modification to different degrees. Possibly the term modification should be left to describe a rather nebulous, but none the less real, condition which has resulted from the transformation of endospermic constituents to give the best possible material for mashing.

The great advances made in recent years in the understanding of biochemical reactions involved in malting may distract attention from the fact that there are many important natural processes (including germination) which cannot yet be adequately described in biochemical terms. When all the individual pieces which make up the jigsaw of germination have been characterized then, possibly, the phenomenon will be able to be described exclusively in biochemical terms; at present, this is not possible. It is, however, still useful to assess the overall results of metabolic changes and, taking into account biochemical transformations which are believed to underlie some of these changes, to consider critically the effects on malting of variables which have traditionally been explored in studies of plant physiology - time, temperature, incident light, water status of the plant, growth stimulants and inhibitors, gas composition of the environment and interactions between different parts of the organism. A physiological survey records the resultant of many integrated biochemical and biophysical reactions and it is valid when these reactions are

imperfectly known or even unsuspected. In what follows many established facts will receive speculative interpretation; extending the metaphor used earlier, it may well be that individual pieces of the jigsaw puzzle are transposed or distorted or inverted and it is certain that some pieces are missing. It is hoped that this review may focus attention on the gaps in knowledge - and stimulate others to try to fill these gaps.

GERMINATION

Viability

The first essential prerequisite for germination is that the grain should be alive. To determine the percentage of viable corns in a sample the only absolutely unobjectionable procedure is to ascertain how many grains will germinate, in optimal conditions, after any tendency to dormancy has been counteracted. A little thought exposes the circular reasoning inherent in this statement: if the seed is alive it will grow, and if it grows it can confidently be said to be alive - but there seems at present to be no way of avoiding it.

Either grain dormancy, or the inadvertant provision of sub-optimal conditions for germination, both of which will be considered later, can cause irregularities in tests depending on growth-tests, which in any event, are slow to yield results. These difficulties have been largely overcome by the use of biochemical tests of viability. The ability of the embryo to reduce tetrazolium salts

correlates well with germinative capacity; either 2,3,5 tri-:phenyl tetrazolium chloride[52] or the more recently introduced 2-(p-iodophenyl)-3-(p-nitrophenyl) tetrazolium chloride,[10] which gives a result in approximately 30 min., can be used with con-:fidence for most samples of grain. The reduction is due to dehydrogenase activity, with the tetrazolium salt acting as a hydrogen acceptor and being reduced to a coloured, non-diffusible formazan. The results of this histo-chemical test, which has also been used to locate sites of succinic dehydrogenase activity in animal tissues,[93] correlate well with standard tests of germinative capacity provided that the grain has not previously been subjected to slight overheating.[10,57] If gross overheating has occurred during storage of moist grain after harvest then dehydrogenases are inactivated, but there is a narrow range, which depends on length of exposure to high temperature and on moisture content of the grain, in which results predicted by tetrazolium tests are much higher than the true germinative capacity. Only rarely do commercial samples experience treatment which brings them into this range, but its existence can be demonstrated by heating moist grain and testing samples at intervals by both tetrazolium and growth tests. In maize, frost-damaged grain may also give anomalously high results with tetrazolium tests.[35] It follows, therefore, that presence of active dehydrogenase is not alone sufficient for viability and that other systems which are implicated

in germination may be more vulnerable to adverse conditions. It is reasonable to postulate that these include the ability to maintain, and increase, dehydrogenase and other enzyme systems which are presumably ultimately under nuclear control. Thus, although catalase activity *per se* is not obviously related to germinative capacity, grain which has been steeped shows an increased value for catalase activity if it is viable, and a decline if it is non-viable.[29] If this is a particular case from a general state of affairs then retention of viability must be dependent on the coupling of respiration with growth, and impairment of this complex pathway cannot yet be detected by any rapid test.

It has long been known empirically that a decrease either in water content of the grain, or in storage temperature, prolongs the life of cereal seeds, and attempts have been made to formulate a mathematical expression, based on temperature and grain moisture, which could predict the life-span of stored seeds. Although a recent comprehensive survey by Roberts[89] has led to an equation for predicting the 'half-viability' of grain (*i.e.* the length of time which will elapse before 50% of the corns have lost their ability to germinate) this type of work, which is based on statistical treatment of records of longevity, does not shed any light on the possible causes of senescence and death. Depletion of respiratory substrate, or of free amino acids, is not apparently responsible for

loss of viability in aged grain, nor is inactivation of certain hydrolytic enzymes,[58] which are still easily detectable in old samples of non-viable barley. To go further than this in discussing the reasons for loss of viability is to enter the realms of speculation.

In what has been said above the barley grain has been considered in isolation, as if it were a sterile structure. This is manifestly not so, and the retention of viability in prolonged storage may be associated not only with the moisture content of the grain but also, simultaneously, with the presence of micro-organisms. Murphy,[68] in 1905, examined samples of barley which had been stored for five years at moisture contents between 12% and 16% and noted that, broadly, viability fell with increasing moisture and that only in the samples stored at the higher moisture levels was there a pronounced mouldy smell and a complete loss of germinability. Grain stored for 5 years at 12% moisture gave 99% of germinable seeds and was free from any mouldy odour.

It is difficult here to distinguish between cause and effect, but Bishop[7] has explored the problem a little further and has demonstrated the presence of mycelium on the inner skin of the grain, though penetration of the embryo was not apparent until some time after the grain had died. Bishop also suggested that metabolic products of fungi, such as acetaldehyde or ammonia, might have a deleterious effect on the embryo and he showed that these

two compounds produced symptoms in barley, such as aborted roots, which resemble those commonly found in deteriorating grain. With the methods then available, Bishop was not able to detect evolution of toxic compounds from the micro-organisms: nevertheless, the potentially toxic action of fungi in grain maintained at moisture contents above 15% should not be overlooked.

Dormancy

The survival value of any mechanism which prevents all the seeds of a wild plant from germinating immediately is obvious and the potential nuisance value of such a mechanism in an economic crop like malting barley is equally apparent. A period of post-harvest dormancy may be imposed on seeds by various different transient physical or chemical barriers to germination which have been incorporated in the physiological make-up of the seed,[67] and dormancy is terminated when these barriers have been broken. The sum-total of the changes which take place while the seed is emerging from dormancy is sometimes referred to as after-ripening, or maturation.

For barley, the first co-ordinated study of problems posed by dormancy was sponsored by the Institute of Brewing Research Scheme in the 1940's and carried out by L.R. Bishop. The resulting memoranda on barley germination (Bishop[5,6,7,8]) set the problem in the wider context of grain storage and seed dormancy in general, and they have formed the background to much subsequent work carried

out by others.

The assessment of dormancy requires a knowledge of the proportion of living seeds which will eventually germinate after maturation; for malting barley this is termed the Germinative Capacity (G.C.) of the sample. The germinative capacity can be estimated by tetrazolium staining or, more accurately but also more tediously, by modified growth tests. The extent of dormancy is measured by incubating replicate samples of 100 corns at 18°C. for three days on two layers of filter paper, supported on 9-cm. petri dishes and supplied with 4 ml. of water. The percentage of corns which germinate in this time is the Germinative Energy (G.E.) of the sample. The corns which do not germinate are either dormant or dead; if the tetrazolium staining gives a figure approaching 100% viability, it can generally be assumed that the ungerminated corns are dormant.

Workers in fields of research other than malting have used different criteria to assess dormancy: thus, obviously, growth in compost for a rather longer time may give more meaningful results for agricultural purposes. The different procedures used have led to some controversy, and in the recommended methods of the Institute of Brewing[44] it is emphasised that the conditions employed in the test be stated, in parenthesis, with the summarized result. Although it is not insisted on in the Institute method, it would also be desirable to quote mean and standard deviation of

replicates of tests for dormancy as it is possible to draw unwarranted conclusions from tests in which insufficient replication has been performed and to assume that, for example, figures of 50% and 60% represent different conditions of maturity when in fact they do not differ significantly.

Light in relation to dormancy - A type of germination control which is not infrequent and which has been fully studied in lettuce seeds[14] is the requirement for imbibed seeds to be exposed to light. The effective wavelength is at the red end of the spectrum (660mμ) and the chain of reactions set in motion by illumination can be reversed by treatment with far-red light, of wavelength 730 mμ. A controlling agent here is the pigment phytochrome[94] which can exist in two forms (P_{670} or P_{730}) The status of phytochrome depends on light: in darkness P_{730} (the species of phytochrome which is formed on illumination) slowly decays to P_{660} and in far-red light the transformation is rapid. In lettuce, therefore, phytochrome must be in the P_{730} (red illuminated) condition for the initiation of germination though other seeds which germinate optimally in darkness[67] depend on a different phytochrome-controlled mechanism. Dependence on the presence of the appro-:priate form of phytochrome is not, strictly speaking, synonymous with dormancy, but recent work suggests that in barley, which is indifferent to prevailing light conditions after it has emerged from dormancy, the germination of dormant grain may be influenced

by the type of illumination provided. In this connection, Burger,[21] working with two cultivated and three wild dormant barleys, found that germination was strongly inhibited by far-red light; as they recovered from dormancy the grains became insensitive to the type of illumination provided. Dormancy would thus seem to be a prerequisite for light sensitivity in barley. However, when dormancy was relieved by treatment with high concentrations of gibberellic acid the far-red inhibition was not completely overcome. Burger interprets these results as indicating that light sensitivity and dormancy are not associated with the same metabolic site in the grain. His results are also consistent with involvement of phytochrome in the germination of mildly dormant barley. This observation is of interest in that the very intensive work at present being carried out on the participation of phytochrome in other phenomena, such as initiation of flowering, can be expected soon to yield biochemical results which may be equally relevant to events occurring during dormancy.

Temperature relations - In many seeds the elimination of dormancy requires a period of storage at low temperature.[67] For response to occur the treated seeds must first have been supplied with water and substantial metabolic changes may take place during the treatment. Thus, there are increases in the nitrogen-and phosphorus-containing components of cherry embryos[70] maintained at 5°C. though no changes are detected when storage is at 25°C.

Again, in ash seeds[104] cold storage allows the formation of a chromatographically-separable growth stimulant which apparently anulls the effects of an endogenous inhibitor. These responses at approx. 5°C. are well documented, though they are certainly not well understood, and the effects of alternating temperatures and possible interactions with different light treatments introduce further complications into an already complex situation.

With barley there appears to be no absolute requirement for low-temperature after-ripening, though freshly harvested seed may show a low maximum temperature for germination (10 - 15°C.); this maximum rises by approx. 10°C. after a few weeks' storage.[1] If this is generally true then growth tests which are performed at 18 - 21°C. could give misleading results for freshly-harvested grain, which would be classed as dormant when in fact it has simply been subjected to adverse growth conditions. This appears very clearly in the results of a comprehensive study of barley dormancy presented by Union & Chapon.[100] During an investigation of threshing damage, Caldwell[22] found that a barley in which one-quarter of the grains failed to germinate in 7 days at 21°C. showed 70% germination after the grain had been stored, moist, at 5°C. for 3 days. Wellington[109] also has performed germination tests on samples of barley received between August and October and tested with and without prior chilling. His results show that, after chilling, 82 of the 138 samples examined gave values of over 90%

germination whereas only 28 of them attained this level without previous cold treatment. Essentially similar response of rice to chilling has been demonstrated by Roberts.[90] There thus seems to be a reasonable amount of evidence to indicate that a period of exposure to low temperature may help to eliminate dormancy if the grains have first been allowed to absorb water. Cold storage of dry grain, on the other hand, prolongs dormancy.

At the other extreme, the beneficial effects of elevated temperature on subsequent germination are well attested, and, in this case, the dry grain responds. On at least one occasion, however, the improvement in germination characteristics which followed heating at 40°C. was attributed to the desiccation,[107] which normally takes place in grain held in open vessels for a few days at elevated temperatures. However, both Essery & Pollock[31] and Hewett,[41] by drying grain without heat and heating grain in circumstances where little loss of moisture was possible, have shown that high temperature and not the desiccation of the tissues, is the factor responsible for ameliorating the germination characteristics. Once again, rice[90] and also wheat,[2] respond to high-temperature storage by more rapid recovery from dormancy.

It appears, therefore, that dormancy may be at least partially eliminated either by exposing the grain, moist, to a temperature of 4°C. for a few days or by holding it, dry, at approximately 40°C. for 3 days. No simple explanation can be advanced to account for

these facts and further comment is best deferred until the effects of other factors which are implicated in germination have been discussed.

Water relations - One of the most useful practical findings of recent years has been the recognition by Pollock, Kirsop & Essery[78] of a condition which they term water sensitivity. A figure for Germinative Energy is derived from the standard 4-ml. growth test and another sample is incubated with 8 ml. of water, when a smaller proportion of the grains may germinate. The barley is then said to be water-sensitive, and a rough measure of the degree of water-sensitivity, additional to dormancy, can be obtained from the difference between the percentage germination in 4 ml. of water and that in 8 ml. of water. This type of numerical value ignores the fact that some of the dormant grains may also be water-sensitive. Water-sensitivity, like dormancy, usually diminishes during storage, though it may be of longer duration, and its disappearance is accelerated in well-dried grain.[30] The importance of this phenomenon to the maltster lies in the fact that grain which, in laboratory tests, exhibits water-sensitivity, may none the less be malted successfully[47] if it is given an air-rest (i.e. if it is removed from the steep-liquor) at an appropriate time which may be between 6 and 10 hr. after the start of steeping, when the grain has attained a moisture content of approximately 35%.[55] Water-sensitivity also occurs in wheat[2] but not, apparently, in

rice.[92] The results obtained by Belderok with wheat[2] are of interest in that though the optimum amount of water for maximum germination of dormant grain appeared to be 4 ml. immediately after harvest this figure fell as maturation proceeded, and 2 ml. of water sufficed to give 100% germination in 4 days ten weeks after harvest. No water-sensitivity then persisted. That excess water in the grain (of wheat at least) is not the immediate cause of water-sensitivity, or of dormancy has also been shown by Belderok.[2] He determined rates of water uptake in individual grains and found no significant difference between those which germinated and those which failed to grow. Such a determination would be difficult to perform with barley where the husk complicates the assessment of entry of water to the interior of the corn. However, if water uptake is essentially similar in all conditions of grain, then the term water-sensitivity is possibly rather unfortunate in that it may direct attention away from the real cause of the failure to germinate.

The covering layers of the grain - A ripe barley grain is invested in a husk, formed from the lemma and the palea which enclosed the flower. That the husk has no discernible effect on dormancy was emphasized by Bishop;[5] nor does the husk influence the extent of water sensitivity. Below the husk is the pericarp-testa (or inner skin) a layer which is derived from two discrete structures which were originally present in the flower. The ovary wall is

transformed to the pericarp, and, as the grain ripens, the epidermal cells of the pericarp degenerate and it becomes fused to the underlying testa. The testa is derived from the inte-:guments which initially enclosed the ovule: the outer integument (adjoining the pericarp) rapidly degenerates but the inner integument which in immature grain appears as a 2-cell deep structure, persists and forms a thick cuticle on both surfaces. At ripeness, the testa consists of two layers of cuticle, and between the two layers there is degenerate cellular material. The testa and the pericarp cannot be separated from one another by dissection.

Adrian Brown,[17] in 1907, first recorded the semi-permeable nature of this layer which, he noted, allowed the passage of water from a 36% solution of sulphuric acid, so increasing the normality of the acid outside. He found that the testa was generally impermeable to inorganic ions, though it permitted the passage of iodine from a 1% solution in potassium iodide. Brown's findings have been amply confirmed by Collins[25] who was able to construct a working osmometer from pieces of pericarp-testa, and by R. Brown[19] who concluded that soluble absorption depended on the size of intermolecular spaces which exclude large organic molecules such as those of sugars, and on electrical forces in the cuticle, which oppose the entry of ionised material. This semi-permeability certainly differs from that of the plasmalemma of a parenchymatous

cell, as it persists in boiled grains of barley.[17]

If the pericarp-testa is removed from the corn, especially from the vicinity of the embryo, dormancy[38] and water-sensitivity[78] are abolished. The impediment to germination must therefore be associated with this double layer. Immersion of grain in 50% sulphuric acid for 4 hr. also abolishes dormancy and water-sensitivity.[76] Examination of sections of acid-treated grain shows that the pericarp is removed, though the testa, protected by the outer cuticle, is not visibly affected, a finding which suggests that the pericarp is the layer which imposes dormancy on the corn. This may indeed be so, but although no visible structural change in the characters of the testa can be detected some other, more subtle, modifications may have occurred. As the cuticle is thinner in the region of the testa covering the base of the embryo and as this region (the locus of the micropyle) is the area at which water uptake is most rapid, some further consideration of its constitution is desirable.

The micropyle - At flowering the two integements which later form the tests are not continuous over the entire ovule; a pore is left at the base and through this pore the pollen tube grows to deliver the male gametes to the ovum and the fusion nucleus. As the seed ripens the micropyle region alters. The cuticle forms in a similar fashion to that described above for the main body of the testa but it is thinner and the two layers of cells become irregularly corky;

according to Krauss[51] there may be some slight lignification on the inner aspect of the corky deposit. Krauss also suggests that the loose packing of the corky cells of the layers near the micropyle may facilitate entry of water in this region.

Experience with grain which has been de-husked by acid indicates that excessively long immersion in the acid causes damage first to the coleorhiza and then to the root tips which lie immediately beneath the micropyle; the area surrounding the micropyle is apparently more vulnerable to acid than are other parts of the testa. When dormancy is eliminated by optimum conditions of treatment with acid, it may be that changes in the micropylar region are the important ones, rather than the removal of the pericarp.

Microflora of the grain - Several years ago Bishop[9] drew attention to the possible intervention of micro-organisms in grain maturation. Obviously, the structure of the fruit of barley is such that air-borne fungal spores are liable to be trapped on the pericarp when the glumes are open and, with the gradual degeneration of the outer pericarp, these organisms would find a medium suitable for growth. In wheat, saprophytic fungi are present in the outer layers of all samples except those which have been grown in arid regions under irrigation,[43] and it is to be expected that mycelial growth would be more extensive in the damp harvest conditions which are also associated with subsequent dormancy. Bishop suggested that species of Aspergillus[9] which can grow in an environment containing only 16% of moisture

may have a dual effect on dormancy: initially the strongly aerobic mycelium utilizes oxygen so preventing its ingress to the oxygen-requiring tissues of the embryo and, with time, the metabolic activities of the fungi may cause partial weakening of the testa so facilitating oxygen penetration. This suggested effect of fungi does not seem to have been explored further, though it certainly merits experimental investigation.

The activities of bacteria have also been invoked as a cause of water-sensitivity in barley, and Blum & Gibbert[13] have claimed that surface sterlization with hypochlorite or mercuric chloride may almost overcome water-sensitivity. The interpretation of these results has been challenged by Jansson, Kirsop & Pollock[46] who pointed out that in the standards set up to assess germination of the water-sensitive grain a preliminary one-hour steep in water had been introduced and this treatment had caused an additional decline (in the 8-ml. test) from 67% to 22% germination - a decline which was apparently annulled by use of antiseptics in the steep. Jansson et al.[46] were unable to relate bacterial counts in the sur-:rounding fluid to water-sensitivity and they found no real amelioration of germination in excess water after treatment with the agents used by Blum & Gibbert. Since loosening the pericarp-testa over the embryo eliminated water-sensitivity without lowering the number of micro-organisms whereas growing grain in running water resulted in a minimal microbial population without any

improvement in germination, Jansson *et al.* deduced that the influence of micro-organisms on germination of water-sensitive grain was a minor one. They may be correct, but it could be suggested that bacteria in the ambient fluid are of much less significance than are those present inside the grain, on the surface of the pericarp; organisms in this region, under the husk, might well survive and proliferate *in situ* even in running water.

In a more recent report, Blum *et al.*[12] have reiterated their conviction that antiseptics can counteract water-sensitivity and that micro-organisms are implicated in this phenomenon; they also contend that the concentration of mercuric chloride used by Jansson *et al.* was high enough to damage the barley and so give misleading results in subsequent germination tests. Clearly there is room for more study of the role of micro-organisms in the germination of water-sensitive grain; the last word on this question has not yet been written.

One useful by-product of this controversy has been the reminder by Jansson *et al.* of the potentially harmful effect of even brief spells of immersion in water on the behaviour of barley in the laboratory germination tests. With one sample, immersion for periods between 1 min. and 1 hr. caused a decrease in germination in 8 ml. of water from 71% to approximately 35%, an effect which has been called "steeping injury" and which is apparently at least partially overcome by addition of suitable antiseptics.

We have now reached the unfortunate position of having three apparently separate contributory causes of failure of viable dormant grain to germinate in conditions which allow complete germination of the same material after maturation. There is dormancy, or failure of grain to exhibit its full germinative capacity in optimal con-:ditions of water supply. To this may be added water-sensitivity which imposes a further restriction when the amount of water supplied is supra-optimal, though not sufficient to be inhibitory to mature grain, and, finally, there is the "steeping injury" noted above. Physiologically speaking, all are really different facets of dormancy; water-sensitivity and steeping injury are examples of secondary dormancy which has been induced in the grain by exposure to adverse conditions of water supply.

Dormancy as a genetic characteristic.- It has long been known that certain cultivars are more prone to exhibit dormancy than are others grown in the same season in the same locality. Thus, the old Scotch Common barley had so little tendency to dormancy that, on adverse seasons, it was liable to sprout in the ear;[7] Domen barley behaves similarly.[102] At the other extreme, the high-yielding American cultivar Trebi showed such pronounced dormancy (less than 10% germination at a time when Peatland gave 100%) that it was wholly unsuitable for malting. From the collaborative tests carried out by the Barley Committee of the E.B.C. up till 1956[11] it became clear that Busser showed the highest dormancy, at 18 European centres, of

all cultivars then under trial. In the 1964 trials,[103] dormancy was not extensive but Ariel was troublesome in certain centres.

In a barley breeding programme, therefore, this liability to prolonged dormancy must be considered in making selections at F_2 and subsequent generations and it has been suggested[7] that cultivars like Scotch Common could be used to introduce genes for minimal dormancy. It would aid breeders considerably if the tendency to dormancy could be shown to be linked with some easily assessable anatomical character which could be identified in a very small sample of grain. Little experimental work seems to have been performed on barley with this end in view, though Wellington's work with red and white skinned wheat[108] illustrates the type of study which might be rewarding.

Whether genetical factors contribute to water-sensitivity has been less extensively investigated, though some results of Pollock[73] suggested that water-sensitivity may be particularly prevalent in Rika and Herta. The number of trials was, however, small.

As the results of the E.B.C. trials demonstrate,[11, 102, 103] the genetic component of dormancy is frequently over-ridden by environmental contributory causes. Thus, the cooler northern maritime stations record dormancy in seasons when even the most dormancy-prone cultivars elsewhere are of high germinative energy three weeks after harvest. A similar situation occurs with wheat, and Belderok[4] has shown that there is an inverse relationship between what he calls the temperature sum (day x excess of temperature above 12.5°C.

during the period of mealy-ripeness) and length of the subsequent dormant period. Use of the relationship not only predicts dormancy for different cultivars, but also gives warning of liability to sprout in the ear. Derivation of a similar type of relationship might be of interest for barley.

Techniques for overcoming dormancy and water-sensitivity - With the passage of time, stored grain normally attains maturity so that, in a few weeks, or at worst, a few months, germinative energy attains the same level as germinative capacity. Various artificial treatments have been examined to determine their ameliorating effect on germination and, though not all are applicable in malting practice, they are valuable in providing pointers to the cause of difficulties in germination.

Reference has already been made to the beneficial effects of storage at elevated temperatures on recovery from dormancy and to the fact that water-sensitivity disappears more rapidly from well-dried barley. No satisfactory explanation of these facts seems to have been advanced. Some chemical treatments which improve germination of barley are listed in Table I where it can be seen that different responses may be recorded according to whether the grain is being examined for simple dormancy or for additional water-sensitivity.

Oxygen supplied directly as the pure gas or indirectly by adding an appropriate concentration of hydrogen peroxide[78] annuls water-sensitivity. There are many records of the successful use of

TABLE I

Effect of Certain Chemical Treatments on Barley Germination*

	Optimal Supply of Water		Excessive Supply of Water		
	Germination (%) in:		Germination (%) in:		
	Water	Test Material	Water	Test Material	Reference
Oxygen (gas)	15	18	-	-	78
Oxygen (gas)	-	-	55	96	78
H_2O_2 (0.5%)	61	59	15	38	78
H_2O_2 (0.5%)	95	95	24	92	78
H_2S (0.5%)**	38	100	15	17	77
Thiourea (1%)**	20	75	-	-	77
$NaNO_2$(0.5%)**	25	88	-	-	74

* Only trials in which specified amounts of water were used are listed here. Further results of this type will be found in Ref. 5.

** The test solutions were applied in a preliminary steep, and the subsequent germination tests were adjusted to allow for the water absorbed.

hydrogen peroxide to overcome dormancy[98] though possibly in the earlier trials the adverse effects of excessive quantities of water were not always appreciated. Although the dormant sample described in Table I failed to show improved germination in hydrogen peroxide this may be exceptional, as personal experience suggests that many genuinely dormant samples do germinate in 4 ml. of water containing less than 0.5% of hydrogen peroxide. Dormant rice, which does not exhibit water-sensitivity, also responds to hydrogen peroxide[91].

The simplest explanation of the beneficial effects of high tensions of oxygen invokes the differential permeability of the pericarp-testa to the gas. Aerobic respiration is essential for root growth, as can easily be seen by incubating excised embryos in nitrogen, and for water-sensitive grains it would appear that any means which ensures entry of oxygen to the embryo will counteract the adverse effects of excess water. The beneficial effects of incubation of freshly-harvested grain in moist conditions at low temperatures could also be ascribed to the greater solubility of oxygen in the outer coverings of the seed.

The restraining influence of the pericarp-testa, even in non-dormant grain, is unambiguously demonstrated by results reported by Robert Brown[20] who compared gas exchange in intact seed with that of isolated embryos during the first 12 hr. of water uptake. He found that, in the intact grain, carbon dioxide output greatly exceeded oxygen uptake giving an R.Q. $\frac{C_{\prime}O_2\cdot}{O_2\cdot}$ of over 3.0 some 5 hrs. after

imbibition began; when the embryo was freed of its restraining pericarp-testa, the R.Q. consistently approached unity. Urion & Chapon[100] have also commented on the period of anaerobic respiration which occurs in the few hours following the moistening of the grain.

However, the results obtained by Urion & Chapon throw some doubt on the validity of any explanation of dormancy based simply on differential permeability. Using Warburg manometry, they found that the uptake of oxygen by barley is of the same order of magnitude whether the grain remains dormant or whether it subsequently germinates. It could be postulated that in the dormant grain the oxygen is utilized by some oxygen-requiring system in the pericarp, so that it does not penetrate to the embryo. It is instructive, however, to consider the implications of Urion & Chapon's findings on the assumption that the supplied oxygen does in fact reach the embryo. We then have an embryo in a situation in which aerobic respiration is possible, to an extent which permits germination of non-dormant grain - but the embryo does not grow. We must now postulate some failure in the synthetic processes which are normally associated with respiration. Dissociation of respiration from synthesis can be achieved artificially by using uncoupling agents, such as dinitrophenol, in whose presence tissue can consume oxygen rapidly but cannot synthesize protein or grow.

It is therefore possible that dormant corns which fail to grow despite the availability of oxygen contain what could be described

in the broadest sense as uncoupling agents which must be removed before germination can be accomplished. Their removal would be impeded by the pericarp-testa, which must act as a barrier to passage of solutes out of the corn, in the same manner as it prevents entry of ions. Removal of the pericarp, or damaging the outer layers of the corn mechanically, may thus allow germination to proceed not primarily by facilitating the entry of oxygen to dormant corns, but rather by removing the barrier to solute diffusion. Pricking the grain, which counteracts dormancy,[78] would also be expected to abolish the osmotic barrier.

Grain which fails to germinate in 4 ml. of water may germinate successfully after treatment with various sulphydryl-containing[77] compounds, including hydrogen sulphide. Pollock & Kirsop[77] have suggested that hydrogen sulphide may be functioning as an inhibitor of phenol oxidases which are present in the pericarp of certain cereals and which would compete with the embryo for oxygen. Belderok[2] has explored this possibility for wheat. He found that, though polyphenol oxidases declined during the two weeks before harvest they remained almost constant thereafter, and he noted that a considerable lapse of time occurred, at constant polyphenol oxidase levels, before germinative energy rose significantly. He concluded that dormancy does not depend on the activities of polyphenol oxidases in the outer layer of the grain.

Working largely with dormant rice, Roberts[92] has confirmed the

efficiency of hydrogen sulphide in eliminating dormancy, and has shown that cyanide has a similar effect; he attributes the action of both to their ability to inhibit cytochrome oxidase. Roberts postulates the presence of a temporary oxidizable inhibitor in dormant rice, he considers that high-temperature storage may promote more rapid non-enzymic oxidation of the compound and he suggests that inhibition of terminal oxidases will temporarily remove a major competitor for the oxygen which eventually destroys the hypothetical inhibitor. As regards the ameliorating influence of nitrite and nitrate, which is contrasted with the inefficiency of ammonium ions, Roberts suggests that these might serve as alternative hydrogen acceptors, so relieving the competition for oxygen in the embryo.

Roberts' theory has the merit of comprehensiveness, though it may be doubted whether cyanide or nitrite can pass through the semi-permeable testa, as he implies; interaction with the pericarp, alone or in association with micro-organisms, would appear to be more probable.

Hormones in relation to dormancy - Pollock and his co-workers[74, 77] examined the effects of the three known natural plant hormones (indolyl acetic acid, gibberellic acid and kinetin) on dormant barley. They found that indolyl acetic acid has no effect on dormancy and, although the lowest concentration used was higher than is normal for physiological work (and indeed was well into the inhibitory range of this compound) other results generally indicate

that the indolyl hormones are not significant in relation to seed dormancy.[33] Gibberellic acid, however, applied at 100 p.p.m., caused a rise in germinative energy from 2% to 65% and kinetin gave an enhancement of similar magnitude. Further results, obtained by the present writer, are given in Table II; these confirm Pollock's earlier observations that gibberellic acid may overcome dormancy and have no effect on water sensitivity.

When Pollock *et al.* supplied the two hormones to non-dormant grain in which the embryo had been exposed to the atmosphere it was found that the speed of growth increased and, moreover, gibberellic acid was effective in improving germination of the very occasional samples of grain which fail to grow after the inner skin has been removed. The hormone thus appears to affect the embryo directly. No adequate explanation has yet been advanced to explain the mode of action of the hormone but it is interesting to note that Frankland & Waring[33] have recorded very similar reactions of dormant hazel and beech seeds to gibberellic acid. These seeds require chilling before they can germinate and gibberellic acid or, better, gibberellin A, is able to replace the cold treatment, provided that the pericarp has been perforated. In dormant hazel seed no endogenous gibberellin could be detected but small quantities appeared after the seeds had been chilled for a few weeks. Growth inhibitors, tested for by a biological method, showed no clear changes during after-ripening, but Frankland

TABLE II

Effect of Gibberellic Acid (25 p.p.m.) on Barley Germination

Cultivar	Percentage of Corns Germinated in 72 hrs.			
	4 ml. water	4 ml. G.A.*	8 ml. water	8 ml. G.A.
Mentor	99	100	20	24
Hunter	100	100	30	39
Ymer	56	98	30	29
Proctor	21	80	8	17
Ymer	83	95	22	14

* G.A. - Solution of Gibberellic Acid.

& Wareing consider that inactivation of an inhibitor may none the less be involved in the promotion of germination by gibberellin. In this connection, the ability of gibberellic acid to overcome the inhibitory action of chloramphenicol offers an interesting parallel.[97] The high concentrations of gibberellic acid required to break dormancy are presumably attributable to the impeding effects of the testa which would be expected to exercise some restraint on the passage of this slightly ionized organic compound.

Endogenous inhibitors and dormancy - Roberts[92] is not alone in suggesting that an inhibitor accumulates in the anaerobic condi-:tions prevailing in mature or nearly mature grass seeds. Vose[106] and Hay[40] have both postulated the presence of a product of anaerobic respiration which is inhibitory in its reduced form and inactive when it is oxidized. Hay further suggests that the reduced inhibitor (in Avena) may be destroyed by oxidases which are activated by wounding. This, he claims, would explain the promotion of germination in punctured seeds - a promotion which is also recorded for dormant barley[78] and for wheat which had been punctured and then sealed to prevent ingress of oxygen.[2] The occurrence of such an inhibitor is merely speculative but if compounds of this type are present, they would be expected to accumulate in greater quantities in circumstances in which the drying of grain in the field is slow - i.e. in the cool damp

conditions which do favour dormancy.

Germination inhibitors recovered from steep liquor include acetic acid and vanillic acid;[26] coumarin derivatives[66] have also been identified in extracts of husks and of rootlets. Although coumarin is a well-known inhibitor of germination, it is impossible to assess the physiological significance of these results as the functional concentration in the embryo is not known.

Changes accompanying maturation of barley - As will be clear from the results discussed above, post-harvest changes in stored barley are such that the grain becomes indifferent to light and to added gibberellin, it can tolerate much greater quantities of water in the germination medium and can germinate over a wider range of temperatures and at a lower partial pressure of oxygen. These are rather generalized changes and it would be interesting to know whether they are accompanied (or possibly caused) by chemically detectable changes in the maturing grain. In this connection, two observations made by Belderok with wheat are of interest. Using a histochemical technique, Belderok found that as grains emerge from dormancy so the protein-bound disulphide groups are transformed to sulphydryl groups[2] - an observation of relevance to enzyme activation - and he also recorded a gradual change in embryo proteins.[3] His results suggest that the synthesis of essential protein may not be complete in grain which is dormant after harvest.

If a comparison can be made, in biochemical terms, between events taking place in imbibed grain which germinates and imbibed grain which, though alive, stubbornly refuses to do so, then very real progress can be expected in the understanding of grain dormancy.

At present we can say that almost all forms of dormancy (including water sensitivity) can be overcome by removing the covering tissues from the surface of the embryo, and so abolishing the osmotic barrier imposed by the cuticle of the tests. Primary dormancy (failure to germinate in optimal conditions of water supply) can be overcome by treatment with gibberellic acid or with hydrogen sulphide, and both inadequate penetration of oxygen and participation of inhibitors may be involved in this phenomenon. Water sensitivity, which is a form of secondary dormancy imposed on some barleys by supplying the corns with excessive amounts of water, appears to be caused by a lack of oxygen in the embryo: the interaction of water and grain tissues - and possibly also micro-organisms - an inducing water sensitivity still requires clarification.

MODIFICATION

It might be thought that a disproportionate amount of this review has been devoted to mechanisms involved in the rupture of the testa by the coleorhiza and to the circumstances which prevent this deceptively simple growth reaction from taking place. However, once germination has occurred subsequent events normally - though not

invariably - proceed without undue difficulty. These events are, at least initially, under the control of the embryo, which forms the seedling plant largely at the expense of the endosperm.

Barleys which have recently recovered from dormancy may be sluggish in growth and may show a slow development of amylolytic enzymes. Moreover, different cultivars give different figures for the "germination behaviour index"[82], the value of which is obtained from the relationship $5x + 3y + z$ where x, y and z are percentages of grain which germinate in 24, 48 and 72 hr. respec-:tively. Both these facets of behaviour are presumably related to metabolic changes in the embryo, and the second at least is genetically controlled.

Cereal embryos excised from dry grains apparently lack the complete machinery needed for incorporation of amino acids into proteins,[65] and hydration of the tissues is a pre-requisite for the activation of messenger ribonucleic acid (m RNA) which completes the system necessary for protein synthesis. By the time the root has emerged, the total RNA in the embryo[42] is increasing, as is protein synthesis. The respiratory substrate utilized include possibly fat (approx. 17% of the dry weight of the embryo[64]) and certainly sucrose and, in aerobic conditions, raffinose (respec-:tively 15% and 5% of the dry weight of the embryo[59]).

Further supplies of nutrients come from the endosperm, and, to make these available, it seems probable that a gibberellin is

secreted by the nodal region of the axis and translocated through the scutellum to the aleurone where it induces formation of hydrolytic enzymes.[63] Products of the action of these enzymes are absorbed by the elongated cells of the scutellum, and seedling growth continues. van Roey and Hupe[101] argued that a check to growth might result from inadequate supplies of sucrose in the embryo, - supplies which might be depleted before enzymic activity in the endosperm was established sufficiently to ensure adequate provision of supplementary nutrients to the seedling. Their results suggested that rapid growth might be correlated with the joint possession of high concentrations of sucrose and vigorous formation of enzymes which hydrolyse endosperm walls: many other metabolic processes, including nucleic acid metabolism, are equally likely to be implicated.

The modification in which the maltster is interested depends on the action of several of the hydrolytic enzymes. β-Glucanases[81,84] and pentosanases[83,85] attack endosperm cell walls, which appear to lack cellulose[62] and to be composed largely of hemicelluloses, and peptidases degrade hordein and other protein reserves; the net result is to produce a friable malt. α-Amylase is also formed in the aleurone[61] and, with the β-amylase already present, it accounts for the increasing diastatic activity of the malt. Inevitably, degradation of some starch occurs, and analysis suggests[36] that during malting this is primarily a β-amylolysis. Phytase action[82]

causes some liberation of free phosphate. Accounts of the mode of action of these enzymes are available elsewhere[39] though information on some of them, notably the peptidases, is less extensive than could be desired.

Water and oxygen - Figures recently presented by Reynolds ~~and~~ & MacWilliam[88] show that at 13°C., there is a rapid absorption of water from the steep liquor by the embryo for approximately 6 hr., followed by a declining rate, to give a level of approx. 58% moisture in the imbibed embryo after 24 hr. The scutellum lags behind the axis of the embryo in its uptake of water, but by 24 hr. the scutellum and the axis are hydrated to the same extent. Distribution of water to the endosperm gives the same type of curve for water content, but at a later time, the rise in moisture bringing the endosperm to 31% water in 24 hr.

These figures are exactly what would be expected from the preferential absorption of water in the region of the micropyle: roots will be hydrated first, the passage of water to the more distant scutellum will be slower, and the endosperm will be reached still later. More sophisticated analysis of the movement of absorped water is injudicious, as the metabolic processes of the grain have a major effect on the moisture content. Respiration in the embryo yields additional water and causes a fall in dry weight and increase in the percentage content of water thus need not imply movement of water into a living tissue. Even in the

non-respiring central endosperm, an apparent loss of water relative to dry weight might be partially ascribed to utilization of water in hydrolysis. Considerations of this type make it necessary to use figures of moisture content rather cautiously.

An interesting side-effect of the increasing moisture content of the grain is seen in the time course of development of hydrolytic enzymes. Formation of extractable amylase,[88] for example, initially rises in parallel with the increase in water content but, unless the grain is removed from the steep-liquor, a decline sets in at about 6 hr. An air-rest at this time allows uninterrupted increase in enzyme activity and, once the grain has chitted it is able to absorb water from a second steep at a rate which increases the moisture content of the grain by 10% in one hour. The requirement for oxygen to maintain enzyme synthesis is not unexpected and measurements made on the content of oxygen in steeping tanks[23] show how rapidly the available oxygen is absorbed by barley.

Intensive aeration, naturally, encourages seedling growth and, although the first desideratum in malting is to secure germination and initiate growth, in subsequent stages excessive growth is an embarrassment. Following the observation by Kirsop & Pollock[48] that development of α-amylase continues satisfactorily in barley from which the embryo is removed after 3 days, efforts have been made to kill or damage the seedling at an appropriate time and to

incubate the endosperm alone for such time as is required to ensure adequate enzyme development and modification. In traditional malting methods, root growth is restricted in the later stages when accumulation of carbon dioxide and drying of the grains impose considerable restraint on growth: the killing of the embryo is thus a logical extension of empirical practice.

The most successful techniques so far developed seems to be a very simple one - that of re-steeping.[75] A suitable schedule for malting with re-steeping may involve a 6 hr. steep at 18°C. (to 33% moisture), 20 hr. in air out of steep, a second 1-hr. steep (to 44% moisture), a second incubation in air for 22 hr., a 1-hr. root-killing steep at 40°C. and a final incubation in air at 18°C. for 40 hr.[54]

Such a procedure recognizes (a) that grain must germinate, and steep 1 and air-rest 1 give adequate moisture and oxygen to ensure this; (b) that further water uptake (steep 2) and oxygen (air-rest 2) are needed for induction of enzyme synthesis in the endosperm; (c) that rootlet growth is economically undesirable (root-killing steep) and (d) that modification can go to completion in the rootless grain. Elevated temperatures are not necessary to kill the roots and in the earlier work[75] a 24 hr. steep at 18°C. was used. It is presumed that the anaerobic conditions of the last steep are responsible for the death - and resorption - of the roots, and it is of interest that the mineral composition of the

water used can affect survival of the roots:[79] distilled water is more lethal to barley roots than a hard water containing 300 p.p.m. of calcium. The treated grain can regenerate roots (if it could not, growth in the field would be impossible after 48 hr. rain) but regeneration is not extensive in the time required, after re-steeping, to complete modification. The success of these laboratory findings is reflected in the fact that malting with re-steeping is now a commercial possibility.[37,99] Other root-killing techniques, including the use of formaldehyde and acetic acid, have been explored with some success.[111]

Temperature - Just as water content and oxygen availability cannot be considered separately, so the effects of ambient temperature cannot be simply related, in isolation, to individual series of metabolic events occurring in the grain. Uptake of water by barley has long been known to be more rapid at higher temperatures[18] and Lubert & Pool[54] have recently shown that induction of water-sensitivity is also greater. The solubility of oxygen in water, of course, falls with rising temperature. Lubert & Pool[54] found that the optimum steeping temperature (as judged by analysis of the finished malt) was that which allowed the Proctor barley which they used to attain most rapidly a moisture content of 32%, provided that the induced water-sensitivity did not exceed about 35%. Slightly different figures were optimal for Olli barley.

This type of compromise between two opposing effects of changing

temperature is likely to be operative in other facets of metabolism. In general terms, it can be stated that respiration has an overall Q_{10} of approximately 2.0 (i.e. with a rise of 10°C. the amount of substrate consumed in respiration is doubled) and values of Q_{10} for other metabolic sequences which are important in malting (e.g. enzyme synthesis in the aleurone, cell wall degradation in the central endosperm, and loss of potential extract to seedling synthesis) could doubtless be ascertained. It might be possible, and it would certainly be interesting - though very laborious even with the aid of a computer - to determine the temperatures for different barley cultivars, at different stages of malting, which gives the best compromise between the various metabolic events which contribute to ensuring a "good" malt, of maximum extract. At present, however, the empirical approach of e.g. Lubert & Pool appears in practice to be more promising - though the understanding of the objectives of malting which we now possess might make it possible to "optimize" malting control.

Hormonal aspects of modification - The use of gibberellic acid is now so well-established that little need be said about general aspects of the use of this plant hormone: a review by Briggs[16] summarizes earlier work on gibberellins in malting, and Paleg[72] has recently discussed the mode of action of gibberellin. When gibberellic acid is added to endosperms, which have been separated

from their embryos, synthesis of the usual range of hydrolytic enzymes proceeds in an apparently normal fashion, though without the hormone, little[71] enzyme formation takes place. Kirsop & Pollock's observation[48] that production of α-amylase continues almost undiminished in grain from which the embryos have been removed after 3 days' growth can now be explained in terms of a gibberellin-like factor from the embryo being mobilized and translocated in this interval of time - and gibberellins are known to be present in barley grains[86]. Yomo[112] and Briggs[15] have demonstrated the existence of material in embryo cultures which has gibberellin-like properties, though it has not been chemically characterized, and embryo-free barley has been "malted" successfully[95,96] by the application of gibberellic acid. It would, however, be unwise to assume that the only significance of the embryo in relation to malting is as a supplier of gibberellin. The living embryo normally forms a 'sink' for amino acids, sugars and inorganic ions liberated from the endosperm, and, in the absence of an embryo, the characters of the malt may be significantly altered. For example, there is a marked fall in pH in endosperm incubated in buffered solutions of gibberellic acid and the increased acidity would appear to extend to the worts[95] prepared from embryo-less grain. The lower pH may also account for the slight drop in amylase activity observed in these worts.

Gibberellic acid exercises its effects through the living

aleurone, it functions only in presence of oxygen, it has an optimal temperature of 30°C. and it shows no effects on endosperm at 40°C.[61] It was suggested by Briggs[15] that α-amylase formation in response to gibberellin is a de novo synthesis, and this suggestion has been confirmed by work reported by Varner and his colleagues,[87,105] who believe that gibberellic acid may act directly on nuclear metabolism. Results of experiments performed with inhibitors of various stages of protein synthesis are consistent with the view that gibberellic acid may function to de-repress a repressor of RNA synthesis in the nucleus; a similar condition may occur in oats.[69]

Successful use of gibberellic acid in malting depends on securing the optimal balance of time of application, temperature, oxygen availability[28] and moisture status[60] of the aleurone to allow the best production of the enzymes and their reaction products which are wanted in the malt, without grossly enhancing other enzymic sequences such as proteolysis, which may not be unequivocally desirable. It is possible to minimize proteolysis, and respiratory loss, by adding potassium bromate,[56] but suitable adjustment of ordinary physiological variables may suffice to give the desired result - and some multi-factorial analysis of malts produced in very different physiological conditions might here prove rewarding.

It is unlikely that gibberellins are the only hormones

implicated in malting, but little useful comment can be made at this time on other plant hormones, or on growth inhibitors, including those present in steep liquors, because so little is known about their mode of action. Possibly when this topic is reviewed again in ten years' time, an as-yet unsuspected growth factor, or growth inhibitor, will have assumed the importance which gibberellic acid holds today.

CONCLUSIONS

The factors of paramount importance in the manufacture of malt include oxygen supply in the embryo and, later, in the aleurone, the water status of the grain, especially in the hours just before germination is accomplished, and temperature relations, which affect different metabolic reactions in rather different ways. All are inter-related - and the importance of all has been appreciated, though possible not understood, since the very early days of malting. That malting technology has advanced over the last fifty years is clear from a comparison of extract yields of today with those obtained at the beginning of the century, and, even more telling, from a comparison of the times required to produce malt from barley. Any further improvements in malting are likely to come jointly from several sources: first, there is the genetical aspect of barley behaviour which, with the availability of micro-maltings[34,110] is much less hit-and-

miss than it was in the pioneer days of Hunter and Bevan; secondly, there is the type of collaborative trial exemplified by the work of the Barley Committee of the E.B.C., which relates malting barley not only to its brewing value but also to the all-important agronomic aspects of production; thirdly, there is the experimental outlook of maltsters who are willing to explore and report on novel malting techniques; fourthly, there is growing knowledge of plant metabolism, which has to be assimilated, and then exploited by the forward-looking maltster; and, finally there might be rewards to be sought in a computerized approach to malting control.

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